Molecular analysis of circadian photosensitivity and diapause in the jewel wasp Nasonia vitripennis

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This research has been carried out at the Groningen Institute for Evolutionary Life Sciences (GELIFES) of the University of Groningen (The Netherlands) and the Department of Genetics & Genome Biology (formerly the Department of Genetics) at University of Leicester, according to the requirements of the Graduate School of Science (Faculty of Science and Engineering, University of Groningen).

This research was founded by the EU Marie Curie Initial Training Network INsecTIME.

Cover design & artwork by Ella Yabsley
Thesis layout: Marcela Buřičová
Printed by: Gildeprint, Enschede

ISBN (printed): 978-94-034-1021-0
ISBN (digital): 978-94-034-1020-3

university of groningen

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PhD thesis

to obtain the degree of PhD at the
University of Groningen
on the authority of the
Rector Magnificus Prof. E. Sterken
and in accordance with
the decision by the College of Deans.

This thesis will be defended in public on

Friday 5 October 2018 at 14.30 hours

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CHAPTER 1

General Introduction

INTRODUCTION

Almost all life on Earth is subject to cyclical changes in the environment (Kauranen et al. 2013). The two main cycles that affect the biosphere are the day-night cycle caused by the Earths rotation around its axis, and annual seasonal change caused by the Earths rotation around the Sun (Meuti and Denlinger 2013). These oscillations have shaped the evolution of organisms since the origin of life almost four billion years ago.

Insects are one of the most diverse groups of animals on Earth. They have adapted to various habitats across latitudes and altitudes, while exposed to different daily and seasonal changes in the environment. They can be active in various parts of the day, showing either diurnal, nocturnal or crepuscular activity (Saunders 2002). As relatively small ectoderms, insects are very sensitive to changes in environmental conditions. As a result, they have evolved the ability to predict upcoming daily and seasonal changes. They can perceive and react to environmental cues that signal cyclic changes in their environment. For example in anticipation of impending winter, insects can react by entering a state of dormancy, referred to as diapause (Košťál 2011). The most reliable cue to anticipate seasonal change is photoperiod. Insects measure the changes in the length of the day/night throughout the year (Saunders 2002). Other environmental conditions such as temperature, humidity and diet can also act as cues. Biotic interactions, such as interactions with other individuals (population density) can also be an indication of seasonal change, but these can vary significantly over time and are less reliable (Saunders 2002).

Adaptation to various cyclical changes in environmental conditions has led to the evolution of intricate time measuring mechanisms. These mechanisms, known as biological clocks, govern rhythmic behaviours over 24-hour or annual rhythms and induce hibernation or diapause. Insects, along with other organisms, anticipate and respond to daily cycling changes via a clock mechanism, called the

"circadian clock", and to seasonal changes with a so-called "seasonal timer" or "photoperiodic clock" (Koštál 2011). Whether or not these two clock mechanisms are overlapping in terms of mechanistic and genetic structure, is subject to much debate.

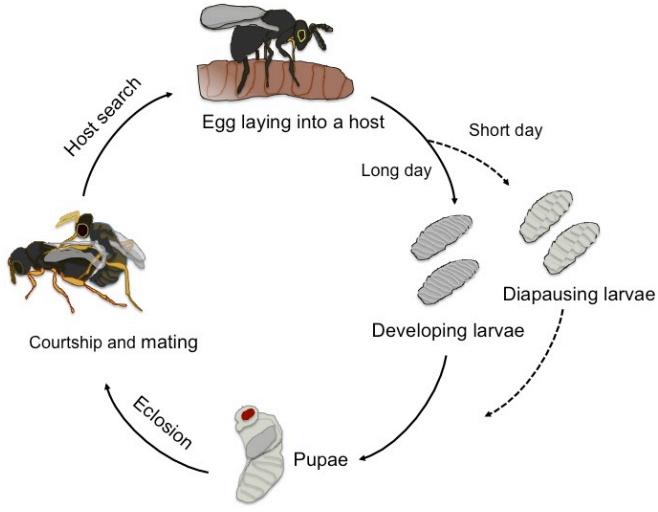
The molecular basis of the circadian clock has been studied for ~60 years in the insect model organism *Drosophila melanogaster*. Less is known about the mechanism in other insect species (Tomioka and Matsumoto 2015). However, thanks to recent advances in molecular techniques and the increased affordability of whole-genome sequencing, non-model species are progressively being studied. Such comparative studies are needed to determine whether *Drosophila* is representative of the wider insect class, and to assess the diversity and conservation in mechanisms underlying the circadian clock. In fact, such studies have already revealed major differences between other insects and *Drosophila* (Rubin et al. 2006; Zhan et al. 2011; Tokuoka et al. 2017), which is consistent with *Drosophila* being a evolutionary derived species (Horch et al. 2017).

Comparative studies between insects other than *Drosophila* will also be very valuable for understanding the evolution of seasonal timers. To provide such a comparative study, I have chosen to investigate the wasp *Nasonia vitripennis* (Hymenoptera) (BOX1), belonging to a group of insects with known differences in circadian clock structure to *Drosophila* (Zhan et al. 2011). The species has been previously shown to have strong light-driven rhythms in behaviours such as locomotor activity and emergence from their hosts (Bertossa et al. 2010; 2013). *Nasonia* also exhibit a seasonal response in the form of photoperiodic diapause (Saunders 1965; 1966; Bertossa et al. 2010; 2013; Paolucci et al. 2013). These characteristics have made *Nasonia* a good model for investigating a link between circadian rhythms and photoperiodism (Saunders 1968; 1974). However the molecular basis of these mechanisms and whether the two are connected is not yet known. My thesis therefore focuses on these issues.

BOX 1 The jewel wasp - Nasonia vitripennis

Nasonia is a genus of parasitoid wasps that belong to the insect order Hymenoptera, superfamily Chalcidoidea, family Pteromalidae. There are four known species: *N. vitripennis, N. longicornis, N. girault i and N. oneida*. *N. vitripennis* is distributed worldwide, *N. longicornis* occurs in western North America, *N. girault i* in eastern North America and *N. oneida* has only been reported from upstate New York (Darling and Werren 1990; Raychoudhury et al. 2010). Hence, *N. vitripennis* inhabits a wide range of latitudes, from the tropics (Marchiori 2004) up to the polar circle (Paolucci et al. 2013).

Nasonia lays its eggs in the pupae of cyclorrhaphous flies, such as blowflies, fleshflies and houseflies. The number of eggs typically varies from 20 to 50 per host pupa, depending on its size. Development from egg to adult takes approximately 14 days at 25°C (BOX 1 Figure 1); eggs hatch after 36 hours, three larval instars are completed within 6-8 days of egg laying, and the pupal stage takes another 6-8 days (Whiting 1967).



BOX 1 Figure 1. Life cycle of *N. vitripennis*.

Like other hymenopterans, *Nasonia* has haplodiploid reproduction with unfertilized eggs developing into haploid males and fertilized eggs into diploid females. *Nasonia* has been the subject of genetic research for more than 50 years, and its genome has been sequenced (Werren et al. 2010). Its total genome size is 298 Mb and consists of 5 chromosomes, containing 12,119 genes coding for 12,988 proteins (Werren et al. 2010). With a recombination rate of approximately 330cm/Kb, recombination in the *Nasonia* genome occurs four times more often than in *Drosophila melanogaster*. Many phenotypic markers such as eye colour, body colour, morphological and lethal embryonic mutations are known and mapped on a linkage map (Saul 1993).

N. vitripennis is a cosmopolitan species that has adjusted to a wide range of environmental conditions. It has a maternal induction of diapause, which means that the mother is sensitive to environmental cues and diapause occurs in their offspring. During long days (> 15 h of light at latitudes 42-52°N) females produce non-diapausing offspring. In short days (< 15 h of light) females start to produce progeny that undergo diapause at the fourth instar larvae stage just before defecation and pupal ecdysis (Saunders 1965; 1966). Once the eggs are in the host puparium, the type of development is fully determined, and diapausing larvae are insensitive to photoperiod (Saunders 1966; 2002). According to Saunders (1965), the maternal induction of diapause in *Nasonia* is affected by three environmental factors, namely photoperiod, temperature and host availability. An additional internal cue of maternal age is also involved in diapause induction. Photoperiodic diapause was studied along a cline in Europe. Latitudinal differences were found between southern and northern populations and this variation was correlated with allelic variation in clock gene *period* (Paolucci et al. 2013; 2016).

The circadian clock in insects

The circadian oscillator consists of alternating positive and negative autoregulatory feedback loops generating a circa 24 h (=> “circadian”, from Latin “circa diem” meaning around a day) rhythm (reviewed in Merbitz-Zahradnik and Wolf 2015). Circadian rhythms typically persist under constant conditions, with a free-running period of approximately 24 hours, but they are synchronised by external environmental cues (Kauraken et al. 2013). Although insects are poikilothermic, these oscillations are temperature compensated, which means that they run at the same pace at various temperatures (Tomioka and Matsumoto 2010). The clock generates a rhythmic expression of clock genes, many of which are evolutionarily conserved (Bell-Pedersen et al. 2005). The timely activity of these clock genes leads to rhythmic biological functions (Bell-Pedersen et al. 2005).

The current model of the circadian clock, which is based on *Drosophila,* consists of three negative feedback loops formed by transcription factors, activators and inhibitors, and is fine-tuned by kinases and phosphatases (Figure 1.1). The first major loop consists of the basic helix-loop-helix (bHLH) and PER-ARNT-SIM (PAS) transcription factors CLOCK (CLK) and CYCLE (CYC) (Williams and Sehgal 2001).Their PAS domains are divided into two structural motifs, PAS-A and PAS-B, the latter being followed by a region called PAC (Huang et al.1993). CLK contains glutamine-rich (polyQ) regions responsible for its transactivation activity (Allada et al. 1998). In the nucleus, CLK and CYC form heterodimers through their PAS domains. This enables their bHLH domains to bind to E-box (CACGTG) enhancer elements in the promoter region of the clock genes *period* (*per*) and *timeless* (*tim*), activating their transcription during the early evening (Darlington et al. 1998). PER, like CLK and CYC, contains PAS-A, PAS-B and PAC domains. The C-terminal region of PER binds to CLK and CYC to inhibit their activity and is therefore referred to as the CLK:CYC inhibition domain (CCID) (Chang and Reppert 2003). The function of TIM is mainly regulating PER stability by forming a PER-TIM complex (in the middle of the night). TIM also accounts for

the light sensitivity of the clock, as it is degraded in the presence of light (Tauber et al. 2007). Phosphorylation of TIM by the kinase SHAGGY (SGG) and phosphorylation of PER by casein kinase 2 (CK2) leads to the initiation of TIM-PER nuclear entry (Akten et al. 2003). This feedback loop leads to the rhythmic expression of *per* and *tim*.

CLK:CYC also regulates the second feedback loop by activation of *vrille* (*vri*) and *Par domain protein one ε* (*Pdp1ε*) expression, which in turn regulates *Clk* transcription via V/P boxes in the promoter of *Clk*. VRI and PDP1 are basic leucine zipper transcription factors able to bind to the same DNA sequence, the V/P box (TTATGTAA), suggesting that they regulate the same target genes. VRI acts as a negative regulator, suppressing *Clk* transcription. Conversely, PDP1ε accumulates later in the nucleus, when it can displace VRI from the V/P sites and activates CLK transcription during the day (Cyran et al. 2003; Glossop et al. 2003).

The third feedback loop is also dependent on CLK:CYC; here the heterodimer activates transcriptional factor *clockwork orange* (*cwo*) (Kadener et al. 2007; Lim et al. 2007). CWO regulates the expression levels of *per*, *tim*, *vri* and *Pdp1ε* (Kadener et al. 2007; Matsumoto et al. 2007; Abruzzi et al. 2011). CWO repress the CLK:CYC mediated transcription in the presence of PER, mainly in the morning phase, as was shown by transcriptional luciferase assay in *Drosophila* S2 cells (Kadener et al. 2007; Matsumoto et al. 2007).

The negative regulatory feedback loop mechanism is synchronised by light via CRYPTOCHROME (CRY). CRY becomes part of a complex with TIM when exposed to light, which leads to TIM proteasomal degradation mediated by the E3 ubiquitin ligase - JETLAG (JET) (Ceriani et al. 1999; Rosato et al. 2001; Dissel et al. 2004; Peschel et al. 2009). As TIM is degraded, unprotected PER is also degraded, after being phosphorylated by a homologue of casein kinase I - DOUBLETIME (DBT), by the E3 ubiquitin ligase SLIMB (Chiu et al. 2008). The activity of DBT is regulated by the kinase NEMO (Chiu et al. 2011). CRY itself is then degraded after light-dependent ubiquitination by another E3 ubiquitin ligase – BRDW3 (Ozturk et al. 2013). Molecular mechanisms of the circadian clock in

insects share many conserved canonical clock genes, but there are also conspicuous differences between species (Figure 1.1). All insects species that were studied so far possess the clock genes *Clk*, *cyc* and *per*, but differ with respect to the presence and role of *cry* and *tim* (Zhan et al. 2011).

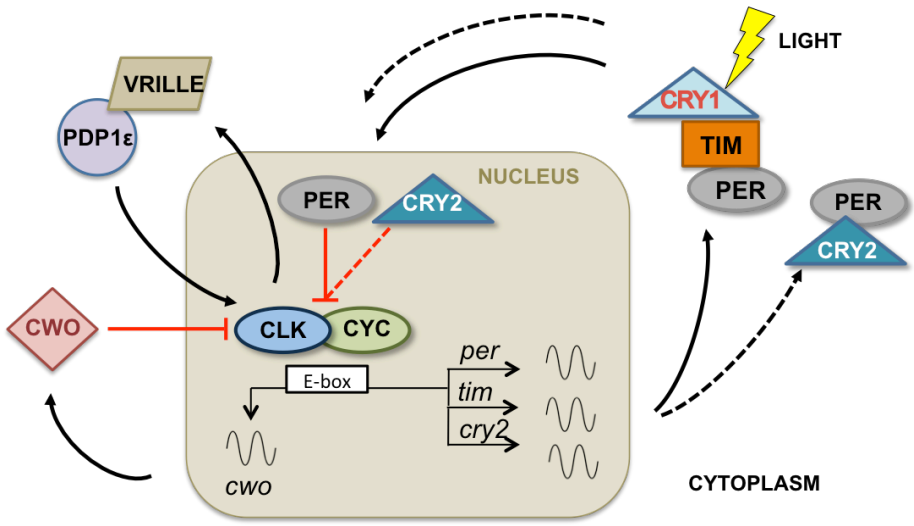


Figure 1.1. Model of an insect clock. Pathways indicated in solid lines are known for *Drosophila,* and the dotted lines are hypothesised for other insect species. See text for details (adapted from Tomioka and Matsumoto 2015).

The circadian clock in mammals

Another well-studied model of the circadian clock is that of the mouse *Mus musculus*. Like in flies, at the centre of the feedback loop are two bHLH PAS transcription factors, CLK and BMAL1, the latter being the mammalian homologue of CYC. CLK:BMAL1 activates expression of *per*, *cry* and *nuclear hormone receptor* (*reverb*) genes. An important difference between the two organisms is the structure and function of CRY. Instead of binding to TIM, as it does in *Drosophila*, mammalian CRYs form a complex with the PERs (plurals are explained below) and

negatively regulate CLK and BMAL1 (Vitaterna et al. 1999; Reppert and Weaver 2001). Recent studies show that the early repressive function of the PER-CRY complex is later substituted by repression from CRY1 alone, independent from PER (Stratmann et al. 2010; Ye et al. 2011; Koike et al. 2012). The stability of CRY is regulated through ubiquitination, mediated by E3 ligase Skp1-Cul1-F-box protein (FBXL3) or FBXL21. Ubiquitination of CRY in the nucleus by FBXL3 leads to its proteasomal degradation (Busino et al. 2007; Godinho et al. 2007; Siepka et al. 2007), whereas FBXL21 labels CRY for degradation in the cytoplasm (Yoo et al. 2013).

In mammals there are two CRY paralogues, CRY1 and CRY2, both of which are considered to be light-insensitive (Griffin et al. 1999), although several studies, mainly *in vitro*, suggest otherwise (Sancar 2003; Hoang et al. 2008; Bazalova et al. 2016). Three paralogues of PER (PER1, PER2 and to a minor extent PER3) are involved in the stabilisation of CRY proteins and their nuclear localisation (Miyazaki et al. 2001; Akashi et al. 2002; Yagita et al. 2002). Mammals lack TIM, but possess a TIM orthologue called TIMEOUT (TIM2), which is also present in flies and other insect species. It has been suggested that TIM2 has a role in light entrainment in *Drosophila*, but its exact function is unclear (Benna et al. 2010). There are thus similarities but also differences in the circadian mechanisms of flies and mammals (Bell-Pedersen et al. 2005), suggesting that the circadian clock has evolved long ago in a common ancestor, but diverged through evolution (Young and Kay 2001).

Cryptochromes

Through insect evolution, the *cry* gene has been duplicated and lost several times. This led to functional differentiation of *cry* into two types of gene families, the "*Drosophila*-like" *cry* type 1 and the "mammalian-like" type 2 family (Yuan et al. 2007). The former is referred to as *cry*1 and the latter as *cry*2 (not to be confused with the similar terminology used for the two mammalian *cry* paralogues). Some

insects, including Hymenoptera, have the “mammalian-like” *cry2*. Some, mainly Diptera, have only the “*Drosophila*-like” *cry1* and some others, like Lepidoptera, have both. Hymenoptera are exceptional for the absence of another core clock gene, *tim* (Rubin et al. 2006; Zhan et al. 2011), and resemble mammals in this respect. This makes the Hymenoptera an interesting insect order to study variation in clock mechanism and possibly seasonal timing. To summarise, insects possess either one or two types of CRY, that have evolved a different circadian clock function as will be described in detail below. As the work here is focused mainly on the *cry* gene in *Nasonia vitripennis*, I will focus on its involvement in the insect molecular clock.

Cryptochromes are flavoproteins belonging to the photolyase/cryptochrome protein superfamily (Thompson and Sancar 2002). Cryptochromes in plants, animals and some bacteria exhibit a high level of gene homology (25-40%), especially in the photolyase homology region (PHR) (Cashmore et al. 1999). Interestingly, the C-terminus shows no homology with photolyases (Ahmad and Cashmore 1993). It varies in length and sequence between species, corresponding to functional changes of the protein (reviewed in Michael et al. 2017).

Photolyases are DNA repair enzymes involved in fixing the damage caused by ultraviolet light (UV, 200 – 300 nm). UV irradiation induces the formation of lesions, which are covalent dimer complexes between pyrimidines on the same strand of DNA. The main two lesions are the dimers cyclobutane pyrimidine (Pyr<>Pyr) and the pyrimidine-pyrimidone (6-4) photoproduct (Sancar 2003). Photolyases recognise these lesions and convert them back to the original structure by cyclical electron transfer. Each lesion type is repaired by a different photolyase, cyclobutane pyrimidine dimer (CPD) photolyase and (6-4) photolyase, respectively (Sancar 2003). Animal CRYs originate from (6-4) photolyases and plant CRYs from CPD photolyase (Mei and Dvornyk 2015).

Photolyases are active during the day, as they are activated mainly by blue light. Photolyases are effective in plants, but not in all species of the animal

kingdom (Selby and Sancar 2006). In placental mammals, the DNA excision repair mechanism has taken over the role of DNA repair (Lucas-Lledó and Lynch 2009). Although photolyases and cryptochromes are evolutionarily related, they have different physiological functions as cryptochromes lack the photolyase DNA repair activity (Thompson and Sancar 2002). Cryptochromes are known to regulate the growth and development of plants, as well as the circadian clock and the magnetic navigation in animals, primarily by absorbing of blue light (reviewed in Michael et al. 2017). Cryptochromes are also involved in the regulation of metabolism in mammals (Lamia et al. 2011; Reddy et al. 2007).

Photoreception by *Drosophila* type CRY1

Drosophila cryptochrome ("dCRY") is a blue-light sensitive photoreceptor that synchronises the clock with light stimuli from the environment (Stanewsky et al. 1998; Emery et al. 2000). It is a non-visual photoreceptor expressed in the clock cells within the brain as well as in the compound eye (Emery et al. 2000; Yoshii et al. 2008; Zhu et al. 2008; Yoshii et al. 2016), although dCRY expressed in the compound eye does not have a significant impact on light entrainment (Yoshii et al. 2015). dCRY is sensitive to light, with even a very short (millisecond-long) stimulus causing long-lasting conformational changes (Ozturk et al. 2009). Light absorption leads to conformational changes in the dCRY C-terminal tail, which regulates the activity of the PHR (Rosato et al. 2001; Dissel et al. 2004). Blue light is sufficient and necessary to cause the binding of TIM and JET to dCRY (Busza et al. 2004; Peschel et al. 2009b).

Transcriptional inhibition of "mammalian-like" CRY2

Light induced structural changes have not yet been reported for CRY2, which was shown to be a transcriptional repressor (Yuan et al. 2007). Therefore, it fulfils a similar role as CRYs in the mammalian-like clock. There are however, some differences in the role of CRY2 between the insect and mammalian models, but

also amongst insects as demonstrated by a comparison between the cricket *Gryllus* and the butterfly *Danaus* (Zhu et al. 2008; Tokuoka et al. 2017). CRY2 in *Gryllus* has six splice-variants, the products of which cannot act as transcriptional repressors by themselves (Tokuoka et al. 2017). Repressor activity was shown only for the variant CRY2c, in the presence of other variants like CRY1 or CRY2f (Tokuoka et al. 2017). This is in contrast to *Danaus*, where CRY2 acts alone as a transcriptional inhibitor (Zhu et al. 2008; Chiou et al. 2016).

Opsin-based pigments

As described above, the photic entrainment of the clock could be mediated via the non-visual pathway by CRY, as is the case in *Drosophila* (Stanewsky et al. 1998). However, the clock also receives light information via visual pathways (Rieger et al. 2003; Hanai and Ishida 2009; Schlichting et al. 2014; Yoshii et al. 2015). In some insect species, such as crickets, the visual path (via compound eye) is the main light-entrainment pathway for the clock (Hamada et al. 2016). This resembles the light entrainment of the mammalian clock, which is mediated via the non-image forming visual system (Nayak et al. 2007). The majority of visual photoreceptors are opsins, belonging to the G-protein coupled receptor (GPCR) family. Opsins bind to chromophores (derivatives of vitamin A), forming a complex sensitive to specific wavelengths of light (Zhong et al. 2012).

Insect opsins

Opsins in insects can be divided into two main classes, the visual rhabdomeric opsins (r-opsins) and the non-visual ciliary opsins (c-opsins)/pteropsins (Velarde et al. 2005; Eriksson et al. 2013). R-opsins differ from c-opsins in their phototransduction pathways, although both use *1-cis*-retinal as the light-absorbing chromophore, and light-induced isomerisation of *11-cis*-retinal to all *trans*-retinal as the first step in the phototransduction process (Fu et al. 2005; Walker et al. 2008).

R-opsin genes occur as four paralogues, coding for different opsin variants. First, three well characterised paralogues: a long wavelength-sensitive opsin (LWS opsin including *Drosophila* Rh1, Rh2 and Rh6) with peak sensitivity in range 500-600 nm, a blue-sensitive opsin (blue opsin, including *Drosophila* Rh5) with peak absorbance at 400-500 nm, the ultraviolet-sensitive opsin (UV opsin, including *Drosophila* Rh3 and Rh4) with peak absorbance at 300-400 nm. Last, Rh7 is a non-visual opsin identified recently in *Drosophila,* that has an unusually wide range of light sensitivity (from UV region up to 500 nm) (Senthilan and Helfrich-Förster 2016; Sakai et al. 2017). It is expressed in the brain, where it functions as the circadian photoreceptor - the first reported opsin in the central brain (Ni et al. 2017). Rh7 plays a complementary role to CRY in the light entrainment of the circadian clock in *Drosophila* (Ni et a. 2017).

R-opsins are suggested to play a role in vision as well as in circadian entrainment, as found in the cricket (Komada et al. 2015). In contrast, c-opsins are not predominantly involved in vision but are believed to play a role in circadian entrainment (Velarde et al. 2005).

Drosophila rhodopsins fall into two groups: the vertebrate-melanopsin-type opsins and the insect-type opsins. Rh3, Rh4 and Rh5 are very similar to insect-type opsins, whereas Rh1, Rh2 and Rh6 are more closely related to vertebrate-melanopsin. However, Rh7 shares only 30% similarity with other *Drosophila* rhodopsins, suggesting that they belong to a yet uncharacterised rhodopsin group (Senthilan and Helfrich-Förster 2016).

The *Drosophila* compound eye comprises approximately 800 ommatidia, independent units each containing eight photoreceptor cells, six outer (R1-R6) and two inner (R7 and R8) (reviewed in Behnia and Desplan 2015). The outer photoreceptors express rhodopsin Rh1, which is a broadband rhodopsin encoded by the gene *ninaE* and is involved in dim light vision and the photoreception of motion (Heisenberg and Buchner 1977; OTousa et al. 1985) The distal inner receptor cell R7 expresses either Rh3 or Rh4, and the proximal inner receptor cell R8 expresses either Rh5 or Rh6 (Rister et al. 2013; Behnia and Desplan 2015).

The distribution of Rh3 in the distal R7 cell and Rh5 in the proximal inner cell R8 creates a pale type of ommatidia (30% of compound eye) and Rh4 expressed in the distal inner cell R7 and Rh6 in the proximal inner cell R8 leads to yellow-type of ommatidia (70% of the ommatidia). These two types of ommatidia are randomly interspersed within the compound eye (Chou et al. 1996; Huber et al. 1997; Papatsenko et al. 1997). Rh2 is expressed in the dorsal ocelli apart from all the other rhodopsins. Rhodopsins are also expressed in the Bolwigs organ in larvae (Yasuyama and Meinertzhagen 1999; Sprecher and Desplan 2008) and the H.B-eyelet of the adult fly (Hofbauer and Buchner 1989; Helfrich-Förster et al. 2001). The compound eye uses histamine and presumably also dopamine and serotonin as neurotransmitters in the light transduction cascade (Rieger et al. 2003; Yuan et al. 2005; Hirsh et al. 2010).

Nasonia possesses three r-opsins: UV-opsin, blue opsin, and LWS opsin (Feuda et al. 2016). In contrast to its relative *Apis mellifera*, it lacks the c-opsin type pteropsin (Davies and Tauber 2016). *Pteropsin* expression in bees is co-localised with brain cells expressing *per* and the neuropeptide encoding gene *pigment dispersing factor* (*pdf),* suggesting a potential role of *pteropsin* in circadian entrainment (Velarde et al. 2005). This, together with the absence of *cry1* and *tim*, makes *Nasonia* an interesting model to study novel light pathways for clock entrainment (both circadian and seasonal).

Vertebrate melanopsin

Light entrainment of *Nasonia* might resemble that of mammals. The mammalian circadian photo-entrainment is effectuated via intrinsically photosensitive retinal ganglion cells (ipRGCs), where the light information is received by an opsin-like molecule named melanopsin, which is involved in non-image formation of vision (Provencio et al. 1998). Melanopsin-containing cell signals via axon projections to the suprachiasmatic nucleus (SCN), the location of the mammalian central circadian clock in the brain (Gooley et al. 2001; Hannibal and Fahrenkrug 2002).

Melanopsin, encoded by the gene *opn4*, is the third type of photoreceptor in the mammalian retina after rods and cones (Provencio et al. 2000). Melanopsin shows the highest sensitivity to light of 480 nm wavelength (Berson et al. 2002; Dacey et al. 2005; Tu et al. 2005). It shares a higher homology with the r-opsins of invertebrate photoreceptors (Gq-coupled visual pigments) than with the c-opsins of vertebrate photoreceptors. Melanopsin signals light through a different phototransduction mechanism (phosphoinositol signalling) than that used in vertebrate rods and cones (cyclic nucleotide signalling), but is similar to Gq coupled visual pigment of insects.

Photoperiodism: a major mechanism for seasonal timing

It appears that the circadian clock was already established in insects that inhabited tropical regions (Saunders 2012). However, photoperiodic mechanisms would have evolved later, as species migrated to temperate regions (Saunders 2012). The mechanism of photoperiodic time measurement consists of four components: (1) light receptors, (2) a photoperiodic timer that distinguishes long nights/short days from short nights/long days, (3) a photoperiodic counter that accumulates with successive long nights/short days, and (4) output pathways that generate various photoperiodic phenotypes (Košťál 2011). The essential part of the photoperiodic clock (genetically predetermined) measures the length of night/day, triggering a response when the critical photoperiod is experienced. The photoperiodic counter accumulates photoperiodic information by counting how many instances of critical photoperiods (CPP) an organism has experienced. Insects have a sensitive period for receiving such information and this period varies between species, developmental stages and populations (Košťál 2011). Both the circadian and seasonal timers depend on light as the main cue to synchronise inner processes with the environment. However, how much photoperiodic components overlap with the circadian clock is still unclear.

Diapause: a pervasive seasonal adaptation in insects

Changing seasons accompanied with harsh environmental conditions led to development of coping mechanisms (Košťál 2011). There are two main categories of dormancy – quiescence and diapause. Quiescence is the direct response of an organism at any developmental stage to any limiting environmental factor. Quiescence allows for activity to re-start immediately after favourable conditions reappear. However, this flexibility is not possible when dormancy occurs as diapause, which is a centrally (hormonally) mediated arrest of development in a species-specific ontogenetic stage, in response to specific stimuli (Lees 1955).

Diapause can occur at different developmental stages specific for each insect species, e.g. embryonic diapause in the Asian tiger mosquito (*Aedes albopictus*), larval diapause in the pitcher plant mosquito (*Wyeomyia smiithii*), pupal diapause in the flesh fly (*Sarcophaga bullata*), and adult reproductive diapause in the linden bug (*Pyrrhocoris apterus*) (Bradshaw and Lounibos 1977; Henrich and Denlinger 1982; Saunders 1987; Lounibos et al. 2003). This diversity in the stage-specificity of diapause (even within an insect order) suggests that the response has evolved multiple times or at least been developmentally modified repeatedly (Meuti and Denlinger 2013).

The environmental cues that regulate diapause (induction and termination) are referred to as token stimuli because they are not directly acting on growth, development or reproduction. They just inform organisms about the risk of continuing direct development. However, how those stimuli are perceived and processed is still largely unknown.

The evolutionary success of insects has for a large part been attributed to their ability to enter diapause (Denlinger 2008). First of all, diapause enables insects to colonize higher latitudes, which probably occurred numerous times (Saunders 2009). During diapause insects increase resistance to a range of environmental stresses, by increasing production of antioxidants, generation of polyols and heat shock proteins, elevation of hydrocarbons in the cuticle and

increase lipid stores (Denlinger 2008). All of these changes in physiology can be considered as adaptations to cope with adverse conditions.

BOX 2 Diapause

Types of diapause

From a developmental point of view, diapause can be classified as either facultative or obligatory. Facultative diapause is a state when insects take an optional decision whether to go into diapause or direct development. Obligatory diapause is a fixed state of development and does not need any token stimuli for induction (Košťál 2011). As organisms are driven to use as much time as possible for reproducing, facultative diapause is more common than obligatory diapause.

From an ecological point of view, diapause can be classified as overwintering diapause (hibernation type) or summer diapause (estivation type) (Masaki 1980). Both types are usually induced by photoperiod, with hibernators reacting to decreasing day length, while estivators respond to lengthening days. Insects that are active during the long summer days and dormant in the autumn are called long-day species, while those that are active during winter diapause are called winter-active (Saunders 2002). For tropical insects, biotic factors such a density of population and food availability, are more critical than photoperiod and temperature (reviewed by Meuti and Denlinger 2013).

Phases of diapause

Insects undergo different developmental and behavioural changes before, during and after terminating diapause. In the following paragraphs, each main phase (pre-diapause, diapause and post-diapause) will be briefly discussed (Koštál 2006).

In the pre-diapause phase, insects receive inducing stimuli from the environment. Those stimuli are received in a specific sensitive period, which is

genetically determined. The sensitive period is species-specific and varies through developmental stages. The point at which half of the population enters diapause is called the critical photoperiod. This response of a given species to a critical photoperiod is plastic and varies across different latitudes and altitudes (Meuti and Denlinger 2013). The induction phase may be separated between developmental stages within the same generation, or between generations. In the preparation phase an organism is already prepared for a later expression of diapause but first undergoes behavioural and physiological changes. Examples include behavioural changes like migration and seeking of suitable overwintering sites, or physiological changes like the building-up of energy reserves (Denlinger 2002).

The next phase is the diapause stage itself, which according to Tauber (1986) is described as: “a neurohormonally mediated, dynamic state of low activity metabolism”. Associated with this is reduced morphology, increased resistance to environmental stress and altered or reduced behavioural activity. Diapause occurs during genetically determined periods of metamorphosis and its full expression occurs in a species-specific manner, usually in response to many environmental stimuli that precede unfavourable conditions. Once diapause has begun, metabolic activity is suppressed even if the conditions favourable for development prevail (Košťál 2006).

The diapause stage is sub-divided into phases called initiation, maintenance and termination. In the initiation phase direct development is arrested, and consequently, metabolic rate is suppressed. Metabolic suppression is a complex process of changes at different levels of regulation, e.g. gene expression, phosphorylation state changes in metabolic enzymes, biological membranes alterations. Some insect species are physically active in diapause, but with a slow decrease in metabolic rate (Košťál 2006). In the maintenance phase, insects do not respond to environmental stimuli even if these are in favour of direct development. During this period insects maintain a low level of metabolism and arrest developmental. With time, intensity of diapause decreases and the sensitivity to diapause-terminating conditions increases (Hodek 1983). In the

termination phase, when the diapause intensity is at a minimum, insects start to respond to environmental conditions signalling that direct development is resumed or restored (Košťál 2006). The final phase, called post-diapause, refers postponement of direct development due to unfavourable environmental conditions (Košťál 2006).

Endocrine regulation of diapause

The hormonal signalling system in insects that responds to diapause initiation and maintenance is well characterised (Denlinger 2012). Types of hormones involved in diapause are species-specific and also vary depending on the developmental stage of diapause induction. The main hormones are ecdysteroids (Ecdysone), juvenile hormone and diapause hormone (Denlinger 2002). Prothoracic gland releases ecdysteroids such as 20-hydroxyecdysone, an active form of Ecdysone, which is a major insect moulting hormone (Maki et al. 2004). Production of Ecdysone is controlled synergistically by prothoracicotropic hormone (PTTH) and insulin-like proteins. PTTH level varies in response to photoperiod and are only effective if an animal reaches certain criteria, e.g. size, weight, etc. (Truman and Riddiford 1974). PTTH is controlled by pigment dispersing factor (PDF), which is regulated by the circadian clock (McBrayer et al. 2007). *Corpora allata* produce a juvenile hormone, a lipid-like hormone, involved in developmental processes, which might also play a role in mediation of the information about the light-dark signal and PTTH levels (Gilbert 2011).

Circadian clock involvement in photoperiodic timing

The photoperiodic timer enables animals to prepare for seasonal changes that will occur in the future, whereas the circadian clock enables animals to face daily changes (Bradshaw and Holzapfel 2010). It was proposed by Bünning in 1936 that circadian clock elements are involved in the photoperiodic timing because both rely

upon the measurement of the length of day or night. Since then, several photoperiodic clock models have been developed, but the exact role of the circadian clock in photoperiodism remains unclear (Kauranen et al. 2013).

The hourglass model (Lees 1973) proposes that the timer consists of a set of biochemical reactions during the dark phase. The process does not require a circadian clock but is driven by external light-dark cycles and needs to be reset every day. The hourglass model fits the photoperiodic response in Lepidoptera (Košťál 2011; Saunders 2011). In contrast, the external and internal coincidence models both involve a circadian oscillator. The oscillators are entrainable by external light, which resets the time measurement during the prolonged dark phases. The external coincidence model (Pittendrigh 1966) assumes a single circadian oscillator. According to Bünning (1936), light has a dual role, both in entrainment and photo-induction. The photoperiodic response, *e.g.* diapause induction by a short day, is produced when the photo-inducible phase regularly coincides with the dark period. In contrast, when the photo-inducible phase coincides with the light period the photoperiodic response is for the long day, *e.g.* direct development (Nunes and Saunders 1999). The damped circadian oscillator model (Lewis and Saunders 1987) is an upgraded version of the external coincidence model. In this model the circadian oscillator dampens in prolonged nights or constant darkness. The internal coincidence model (Pittendrigh 1972) is based on two oscillators, a morning and an evening, and their phase relationship. The length of day responds to phase angle between oscillators, thus the lower the angle, the shorter the light period is, leading to photoperiodic response. The circadian resonance model (Pittendrigh 1972) states that environmental light-dark cycles influence the counting information of a photoperiodic "counter". The idea was expanded by Veerman and Nunes (1987) in their hourglass timer-oscillator counter model. This model combines night measurements by an hourglass-like timer and circadian oscillators are involved in the photoperiodic "counter".

Effect of circadian clock on photoperiodic response

Circadian clocks are ubiquitously present amongst insects, which suggests an ancient and highly conserved phenomenon, of which the diversity is explained by evolutionary radiation of modern insect species (Saunders and Bertossa 2011). If the circadian clock is involved in photoperiodic timing, then an equally diverse array of photoperiodic mechanisms might be expected as well (Saunders and Bertossa 2011).

On a molecular level, the involvement of circadian genes in photoperiodic timing can be viewed in two ways. Emerson et al. (2009) raised the idea of the pleiotropic function of circadian genes involved in the photoperiodic response (Figure 1.2). Pleiotropy means that a single gene can affect more than one phenotype. Alternatively the mechanism could function through modular pleiotropy, where one or more genes affect a whole group of other genes (module). In the case of modular pleiotropy, if a mutation occurs within a clock gene it will change the function of the circadian clock and will have further impact on diapause response. Diapause response can be affected indirectly through photoperiodic input or hormonal pathway, or directly through the clock. In case of gene pleiotropy, when the mutation occurs in the circadian clock, it might lead to altered function of the circadian clock, but it will affect the diapause response independently of its role in the circadian clock (Emerson et al. 2009). The ability to distinguish between modular and gene pleiotropy is necessary for understanding the genetic basis of phenotypes (Emerson et al. 2009).

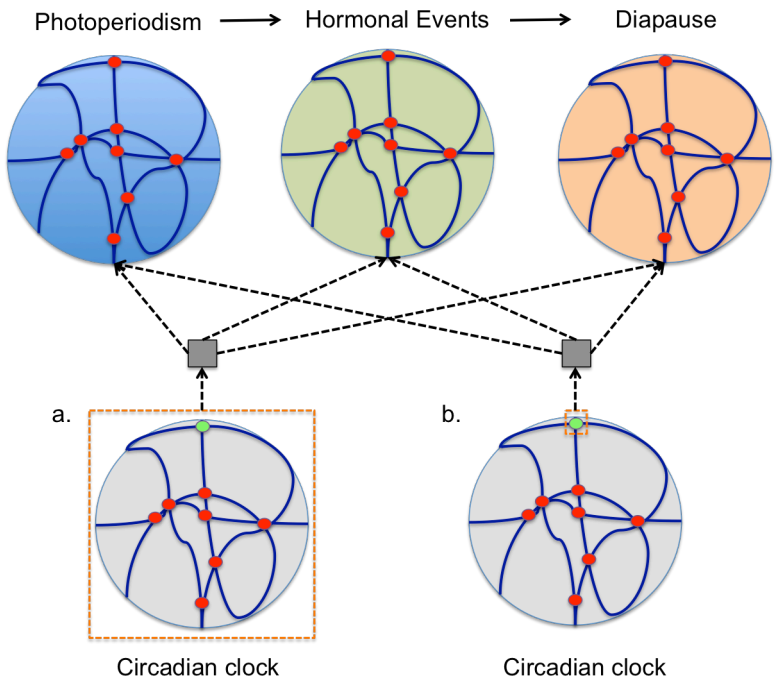


Figure 1.2. Hypothesized effects of circadian clock on diapause. The hypothesis addresses what would happen if a mutation occurred in a clock gene (represented as green dot). Hypothesized effects are (a) Modular pleiotropy (b) Gene pleiotropy (see text for further explanation). The circadian clock (grey) affects diapause through unknown mechanisms depicted as grey box and dotted lines indicating direct influence. Functionally related genes (red dots) are integrated (blue line) into "modules" represented by a circle. The represented successive modules are photoperiodism (blue), hormonal events (green) and diapause (orange) (adapted from Emerson et al. 2009).

Research objectives and thesis overview

The main aim of my PhD thesis was to gain more knowledge about the molecular mechanism of the circadian and seasonal clock of *N. vitripennis*. This information would allow a better understanding of how insects can adapt to daily and seasonal cyclical changes in their environment.

Chapter 2 investigates the functional role of putative clock genes within the clockwork mechanism. In order to study the basic transcriptional-translational feedback loop, I have used several approaches. First, I studied putative clock proteins *in silico*, and compared their functional domains and motifs. Second, I examined the expression level of various clock gene candidates, through a 24 hour time course in light-dark cycle and under constant light conditions, to determine the circadian oscillation of the putative clock genes. Lastly, I used a cell reporter assay to study the negative feedback-loop of the circadian clock of *Nasonia.* The utility of this system was already shown for various insects species (Chang et al. 2003; Yuan et al. 2007).

In Chapter 3, I focus on the gene *cryptochrome2* (*cry2*) and its potential role as a photoreceptor. Behavioural assays were used to measure circadian rhythms of wasps after knockdown of *cry2* under various light entrainment regimes. Knockdown was performed by double strand RNA interference (RNAi). This provided insight into whether light-driven regulation of circadian behaviour is mediated via *cry2*. I created phase response curves for *N. vitripennis* males and females in normal and *cry2* knockdown wasps. The free-running period was measured under constant light and phase shift after a light pulse. Circadian behaviour and light entrainment were measured, at different light wavelengths, to identify the one that provide the most sensitive response. Finally, I assessed the light induced degradation of Nasonia CRY2 in a cell reporter assay.

Chapter 4 investigates the natural variation in circadian rhythms and diapause propensity. I used the *Nasonia vitripennis* Genetic Reference Panel (NVGRP) consisting of 34 natural isofemale lines (van de Zande et al. 2014). These lines have been sequenced and an array of single nucleotide polymorphisms (SNPs) is available to identify genes for specific traits (van de Zande et al. 2014). I carried out a genome-wide association study (GWAS) to identify SNPs (and candidate genes) relevant to circadian and diapause function.

In the final **Chapter 5** I discuss the main findings of my project. I discuss how my results contribute to the field of chronobiology, specifically regarding the clock mechanism of *N. vitripennis* and the role of the core clock gene *cry2*. I recapitulate the current knowledge about light-entrainment mechanisms in insects. I propose a model for the negative feedback loop mechanism of the circadian clock in *Nasonia* and make suggestions for further direction of investigation.

CHAPTER 2

A functional analysis of clock genes in the wasp Nasonia vitripennis

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ABSTRACT

The circadian clock is a crucial component in the regulation of the physiology and behaviour of an organism. The clock architecture in terms of genes and their regulation has been well established in some species. Studies reveal differences in clock architecture between mammals and insects, but also between different insects species. However, too few species have been investigated for a full picture of the variation in clock organisation among insects. This chapter focuses on the functional molecular mechanism of the clock of the hymenopteran *Nasonia vitripennis*. This species has traditionally been used for investigating circadian and seasonal rhythms. However, the functional regulation of its clock is still incompletely understood and how the clock is processing light input is still an open question, as both the light sensitive CRYPTOCHROME1 and TIMELESS are absent. I identified orthologues of clock genes *period, Clock*, *cycle* and *cryptochrome2* and their expression over 24h was profiled by qPCR in a light-dark cycle and under constant light. None of the transcripts, except for *cycle*, showed 24 h cycling in a light-dark cycle. I bioinformatically verified that these genes are more similar to the “mammalian-like” clock genes than to those in *Drosophila*. Based on sequence similarities, it is more likely that *Nasonia* CRY2 is a light-independent regulator of the clock mechanism. A luciferase transcription assay confirms the function of *Nv*CRY2 as a negative regulator of *Nv*CLK:CYC transcriptional activation.

INTRODUCTION

Adaptation to various cyclical changes in environmental conditions has led to the evolution of intricate biological time-measuring mechanisms. These mechanisms, known as biological clocks, govern rhythmic behaviours such as 24-hour rhythms and seasonally induced hibernation or diapause. The circadian clock enables organisms to synchronise with daily changes in environment (Kauranen et al. 2013). The clock has an endogenous rhythm but is entrained by external stimuli such as light, through various mechanisms (Yoshii et al. 2016). Although there are similarities between different organisms in the clock architecture and the light synchronisation of the clock, there are also many differences in gene architecture and regulation.

Molecular clock mechanisms have been studied in detail in the insect model organism *Drosophila melanogaster* as well as in the mammalian model *Mus musculus*. The *Drosophila* circadian clock differs in various ways from the mechanism characterised in mammals, particularly in the structure and function of the genes *cryptochrome* (*cry*), *Clock* (*Clk*), *cycle* (*cyc*) and the presence of *timeless* (*tim*) (Stanewsky et al. 1998; Kume et al. 1999; Yuan et al. 2007; Zhan et al. 2011; Uryu et al. 2013; Gu et al. 2014). Suprisingly, many insect species share more similarities with the "mammalian-like" clock than with *Drosophila*, regarding the presence and function of clock genes (reviewed in Tomioka and Matsumoto 2015). There is thus a need to study other, non-model insect species in order to better understand the evolutionary diversification in adaptation to cyclical environmental conditions.

There are two forms of *cry* that have been identified in insects, referred to as "*Drosophila*-like", *cry1* and "mammalian-like" *cry2*, which are not to be confused with the similar nomenclature used for the two mammalian *cry* paralogues (Griffin et al. 1999). Functionally, the existence of two types of *cry* in insects has led to major differences in clock regulation between species, because *cry1* is a photoreceptor for clock entrainment in *Drosophila* (Stanewsky et al. 1998),

whereas “mammalian-like” *cry2* does not the photoreceptive function and operates as an inhibitor of transcriptional-translational feedback loop in the clock mechanism (Horst et al. 1999; Vitaterna et al. 1999; Zhu et al. 2005; Yuan et al. 2007).

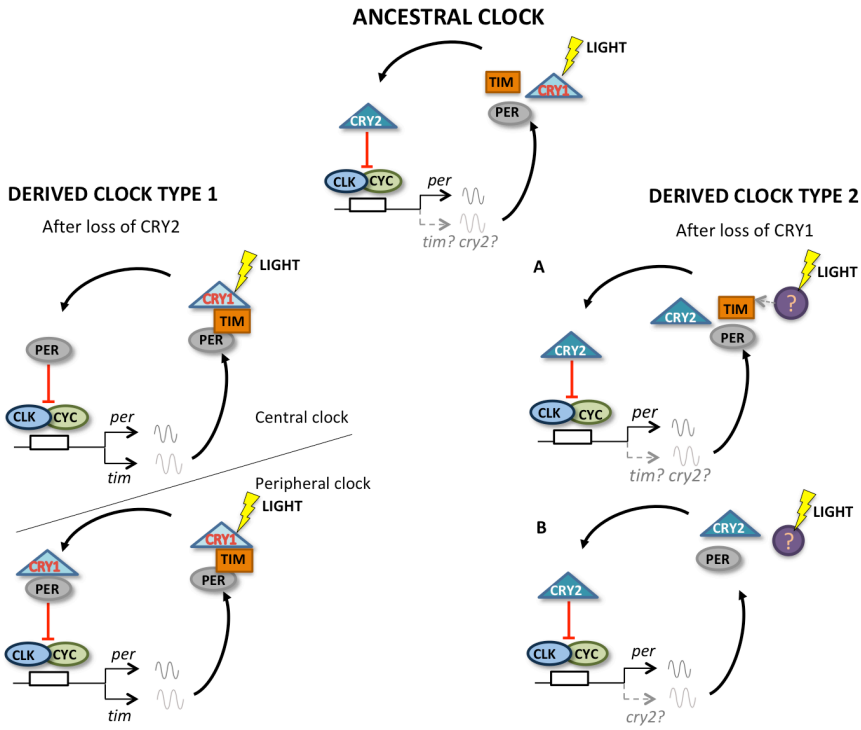

Figure 2.1. Three major types of clock models in insects (adapted from Yuan et al. 2007).

Based on the presence or absence of the two forms of *cry*, Yuan et al. (2007) proposed models for the clock mechanism of different insects (Figure 2.1). The ancestral clock type possesses both CRY1 and CRY2. Each plays a different role within the clock mechanism - CRY1 in photoreception and CRY2 in transcriptional inhibition - as revealed in *Danaus plexippus* (Lepidoptera) (Zhu et al. 2008; Merlin et al. 2013). The ancestral clock diverged into two derived types

following loss of either of the two CRY variants (Yuan et al. 2007). In drosophilids (Diptera) CRY2 was lost, whilst CRY1 acts as a photoreceptor in the master clock. CRY1 also gained an additional role as a negative regulator in the peripheral clock, where it acts together with PERIOD to repress CLOCK:CYCLE-mediated transcription (Collins et al. 2006). Other insect orders lost CRY1 and possess only CRY2, such as beetles (Coleoptera) (Figure 2.1A) and bees (Figure 2.1B) (Hymenoptera) (Yuan et al. 2007). The role of CRY2 in the hymenopteran *Apis mellifera* is a transcriptional repressor (Yuan et al. 2007), but whether this is true of other hymenopteran species remains to be seen. Importantly, in the absence of CRY1 an alternative light input pathway must operate, an issue that I address in subsequent chapters.

The hymenopteran *Nasonia vitripennis* has been previously shown to exhibit strong biological rhythms, both circadian and seasonal (Saunders 1968; Bertossa et al. 2013; Paolucci et al. 2013). *Nasonias* clock genes *period* (*Nvper*) and *Nvcry2* were previously characterised as homologues of other hymenopteran species such as the honeybee (Bertossa et al. 2014). Domains of the *Nv*PER and *Nv*CRY2 proteins showed similarities to “mammalian-like” PER and CRY (Bertossa et al. 2014). *Nvper*, another clock orthologue - *cycle* and the genomic region surrounding *Nvcry2* were also previously associated with photoperiodic diapause induction in *Nasonia* in a QTL study (Paolucci et al. 2016). This work aims to further understand the functional roles and interactions of the putative clock genes within the *Nasonia* circadian clock mechanism.

My main question is whether the clock architecture of *N. vitripennis* resembles the “mammalian-like” mechanism reported from *A. mellifera* (Rubin et al. 2006; Yuan et al. 2007), or whether it is more similar to *Drosophila*. I compare functional domains and motifs of the proteins encoded by the putative clock genes of *Nasonia* to orthologous proteins in other species, expanding on the findings of Bertossa et al. (2014). I measure circadian expression profiles of the *Nasonia* clock genes under light-dark and constant light regimes. The aim of this functional analysis is to extend existing knowledge of *Nvper* and *Nvcry2* transcriptional

oscillations to other putative clock genes such as *NvClk*, *Nvcyc*, *timeout* (*Nvtim2*) and *pigment dispersing factor* (*Nvpdf*), as well as to validate previously identified circadian oscillators such as *allatostatin*, *long wave opsin*, gene *loci 464* and *p450* (Davies and Tauber 2016). Finally, I investigate the functional interplay between putative components of the *Nasonia* circadian clock. Using a luciferase reporter assay, I assess transcriptional regulation of core clock genes, with a particular focus on the role of *Nv*CRY2 as a transactional repressor.

MATERIALS & METHODS

Gene homology analysis

For the purposes of the study I first identified orthologues of the putative clock genes in the *Nasonia* gene assembly with WaspAtlas (Davies and Tauber 2015). WaspAtlas uses a reciprocal best blast hit (RBH) approach, supplemented with data from Ensembl (https://www.ensembl.org/index.html) and NCBI database (https://www.ncbi.nlm.nih.gov/). Protein sequences of homologous genes were obtained from NCBI database. Domain and motif sequences of clock genes were identified with NCBI CDD (https://www.ncbi.nlm.nih.gov/cdd), EMBL SMART (http://smart.embl-heidelberg.de) & MYHITS (http://myhits.isb-sib.ch/cgi-bin/motif_scan). Additional putative domains and motifs were added based on previous publications describing the structures of homologues (Chang et al. 2003; Hirayama et al. 2003; Hirayama and Sassone-Corsi 2005; Rubin et al. 2006; Werckenthin et al. 2012; Bertossa et al. 2013). Protein and domain similarity was assessed by NCBI BLAST, blastp (https://blast.ncbi.nlm.nih.gov) alignment algorithms and EMBOSS Pairwise Alignment Algorithms (EMBL-EBI).

Expression profiling of putative clock genes in light-dark cycling

To test the function of the orthologous clock genes, changes in transcript levels across a 24h light-dark cycle (LD) were assessed by quantitative PCR (qPCR). Newly emerged male wasps were moved to 18°C, LD 12:12 in a light box with light intensity ~ 45 lm/ ft^2. They were collected on day 5 every 3 h, the first collection being one hour after lights on (ZT1 = Zeitgeber Time 1). The wasps were snap frozen in liquid nitrogen, heads were removed by vortexing and for each time point 40-50 heads were pooled; 3 biological replicates were collected and analysed.

Total RNA was extracted with Trizol reagent (Ambion) following the manufacturers protocol. Total RNA was treated with DNA-free™ DNA Removal Kit (Ambion) to remove genomic DNA. Quantity and quality of the RNA samples were evaluated with a NanoDrop 2000 spectrophotometer (ThermoScientific). First strand cDNA was reverse transcribed from 1 μg of total RNA with SuperScript® II Reverse Transcriptase (Invitrogen), using a ratio of 1:6 of Oligo(dT18) Primer (Thermo Fisher Scientific) to Random Hexamer Primer (Thermo Fisher Scientific), according to the manufacturers protocol. Template RNA was degraded with RNase H (New England Biolabs). Controls reactions with no reverse transcriptase (-RT) were performed to assess genomic DNA contamination.

Primers for qPCR (listed in Supplementary table S1.2) were designed with NCBI primer BLAST (Ye et al. 2012) and tested for specificity and optimal binding temperature by gradient PCR. Brilliant® II SYBR® Green Low Rox QPCR Master Mix (Agilent Technologies UK Ltd.) was used for qPCR in 10 μL reactions with 2 μL of cDNA diluted 10-fold post-synthesis. 3 technical replicates were performed per sample. The temperature profile for the qPCR reaction started with activation of DNA polymerase at 95°C for 15 min, followed by 45 sec cycles of denaturation at 95°C for 30 sec and annealing/extension 60°C for 45 sec.

Individual samples were subject to melt-curve analysis to assess amplification specificity. Threshold cycle values (Ct) of negative controls (-RT and no-polymerase reactions) were assessed for contamination by genomic or

exogenous DNA. Ct values of technical replicates were compared and eliminated if differences were greater than 0.5 cycles. Average raw fluorescence of technical replicates was used for each biological sample and analysed using the R statistical package qPCR (Ritz and Spiess 2008). The Ct value and efficiency (E) for individual sample were calculated with sliding window method (Ye et al. 2012). Expression levels for each time point were calculated using formula: $1/ (E_{(gs)}^{Ct(gs)}/E_{(rs)}^{Ct(rs)})$, where gs stands for gene of interest and rs for the reference gene (*rpl32*). Analyses of the circadian gene oscillation were carried out with the statistical software Circwave V.1.4 (developed by R.A. Hut; available from http://www.euclock.org/; see also Oster et al., 2006) was used to determine a fit of a sinusoidal wave with 24 h periodicity upon forward linear harmonic regression for each gene expression data set. The significance levels were determined by F-test, where the fundamental sinusoid wave is tested against a fitted horizontal line through the overall average.

Expression profiling of putative clock genes in constant light

The endogenous cycling of putative clock genes transcripts was examined by measuring mRNA levels, under conditions of constant light (LL) in order to promote free-running of the circadian clock. Samples were collected at two time points 12 h apart, to ensure opposite phases of cycling. The putative clock genes *Nvper*, *NvClk*, *Nvcyc*, *Nvcry2*, *Nvtim2* and *Nvpdf* were investigated as well as genes previously reported to show circadian oscillation, such as *allatostatin* (*Nvallst*), *long wave opsin* (*opsin LW*), gene *loci 464* (*Nvloc 464*) and *p450* (*Nvp450*) (Davies and Tauber 2016).

Wasps were entrained at 18°C, LD 12:12 in a light box of light intensity ~ 45 lm/ ft^2. They were collected on the first day of constant light 3 h (CT3 = Circadian Time 3) and 15 h (CT15) after lights on, and snap frozen in liquid nitrogen. For each replicate 20-30 heads were pooled and 5 biological replicates were analysed. Samples were prepared for qPCR as described above with the

exception that just 0.7 μg of total RNA was used for cDNA synthesis. qPCR was performed and analysed as described above. Difference in the gene expression between CT3 and CT15 were calculated as a mean ratio of control to sample (in our case CT3 to CT15), normalised to the reference gene (*rpl32*). Statistical significance of the ratio was calculated with permutation approach (2000 permutations) with 95% confidence intervals. This approach randomly reallocates Ct and efficiency values between treatment and control samples. The ratio obtained from the original data is compared to the ratio calculated from each permutation and the p-value is calculated based on the number of times the ratio obtained is higher/equal/lower than the original data (Ritz and Spiess 2008).

Sub-cloning and sequence analysis of predicted clock genes

Before assessing the transcriptional regulation of the putative clock genes through the luciferase assay (see below) I had to verify their sequence. First, I sub-cloned the putative clock genes to create cDNA with the CDS regions of predicted clock genes of *N. vitripennis*, namely *Nvper*, *NvClk*, *Nvcyc* and *Nvcry2*. The major variant for each gene was predicted on the basis of its higher abundance in RNAseq expression data reported in WaspAtlas (Davies and Tauber 2015). Primers for amplification were designed in the 3UTR and 5UTR regions to obtain the full coding sequence using NCBI primer BLAST (Ye et al. 2012). cDNA were prepared from RNA extracted from ~30 males (whole bodies) using Trizol reagent as described above. cDNA templates were amplified using Phusion® High-Fidelity DNA Polymerase (New England Biolabs) in a PTC-100 Peltier Thermal Cycler (MJ Research). The PCR cycle was optimised to each gene and individuals primer set (listed in Supplementary table S1.2). PCR products were run on 1% agarose gel and extracted with MinElute Gel Extraction kit (Qiagen). Cleaned PCR products were ligated (blunt end) into a pJET1.2 vector (CloneJET PCR Cloning Kit, Thermo Scientific) or into a TOPO vector (Zero Blunt® TOPO® PCR Cloning Kit, Thermo Scientific) by overnight incubation at 16°C. The ligation reaction was cleaned with

E.Z.N.A.® Cycle Pure Kit (Omega Bio-tek) following the manufacturer s instructions and finally eluted in 15 µL EB buffer. Ligated plasmids were transfected into electro-competent XL-1 Blue *E. coli* using electroporation (Gene Pulser II, Bio-Rad) and plated on LB Agar/Amp plates [100 µg/ mL] for overnight growth at 37°C. Colony PCR was performed to identify positive colonies that were then grown overnight in LB/Amp. The cultures were processed using the E.Z.N.A.® Plasmid Mini Kit I (Omega Bio-tek) following the manufacturers instructions and were eluted with 60 µL of elution buffer. Quantity and quality of the plasmids were evaluated using a NanoDrop 2000 spectrophotometer (ThermoScientific) and the identity of the clones was confirmed by Sanger sequencing (GATC Biotech Ltd.). Sequences were identified using the NCBI website tool BLAST (https://blast.ncbi.nlm.nih.gov/Blast) and aligned using ClustalW (McWilliam et al. 2013) within the software MEGA and Staden Package (Tamura et al. 2007; http://staden.sourceforge.net/).

Cloning and plasmid preparation

To assess the transcriptional regulation between the putative clock genes (*Nvper*, *NvClk*, *Nvcyc* and *Nvcry2*) by luciferase assay, I cloned the genes of interest into a compatible expression vector for *Drosophila* S2 cells. These cells had already been identified as a suitable system to test the basic feedback loop in the circadian clock in other insect species (Chang et al. 2003; Yuan et al. 2007). The luciferase reporter was placed downstream of the PER promoter, because the luciferase assay was based on the hypothesis that CLK and CYC create heterodimers, which in turn will activate transcription of PER. In this system it is also possible to add potential transcriptional repressors such as CRY2 (Yuan et al. 2007).

Genes of interest were PCR amplified from sub-cloning vector into pAc5.1/V5-HisA (Invitrogen). The PCR cycle consisted of denaturation at 98 °C for 30 sec, then 35 cycles consisting of 98 °C for 10 sec, 69 °C for 30 sec, and 72 °C for 90 sec, and a final elongation step at 72 °C for 8 min. PCR reaction was run

with Phusion® High-Fidelity DNA Polymerase (New England Biolabs) in 50 µL volume with pJET primers, forward 5-CGACTCACTATAGGGAGAGCGGC-3 and reverse 5-AAGAACATCGATTTTCCATGGCAG-3. PCR products were run on 1% agarose gel and extracted with MinElute Gel Extraction kit (Qiagen). Quantity and quality of the PCR products were evaluated by NanoDrop 2000 spectrophotometer (ThermoScientific).

Drosophila S2 cells expression constructs (pAc5.1-*Nvclk*, pAc5.1-*Nvcyc*, pAc5.1-*Nvcyc*ΔC, pAc5.1-*Nvper*-V5-His6 and pAc5.1-His6-*Nvcry2*) with a constitutive *actin* promoter from the Drosophila gene *actin-5C* were created. Overlapping primers for cloning were designed using NEBuilder Assembly Tool (http://nebuilder.neb.com/) with an insertion of Kozak sequence (CAAA), for more efficient translation of the gene, in the 5UTR of the start codon of each gene. Additional His6 or V5 and His6 epitope tags were added at N-terminus for *Nvcry2* and C-terminus for *Nvper* respectively. Primers were annealed to genes via PCR reaction adjusted for individual genes and primer sets (Supplementary table S1.2). The deletion ΔC in *Nvcyc* occurred unintentionally during cloning of *Nvcyc*, probably through mis-annealing of primers. Cloning vectors were digested prior to cloning with either *XbaI* and *ApaI,* or *Xba* and *XhoI*. Gel purified PCR product with adjusted primers attached were used in Gibson Assembly® Master Mix (New Englan Biolab) (GA). Assembly protocol for 2 fragments (vector: insert ratio 1:2 in weight) was performed as recommended by manufacturer and consequently transformed into chemically competent cells. Selected colonies were grown overnight in LB/amp and plasmids were purified and sequenced as described above. The bioinformatics software SnapGene (from GSL Biotech; available at snapgene.com) was used to help visualise and design the sequences.

Reporter constructs for the luciferase assay were the pGL3 *4E-hsp-luc*, consisting of *an* E-box *(CACGTG)* in four tandem repeats with 18 bp of immediate flanking sequence, fused with a *hsp70* promoter upstream of *luciferase* (Darlington et al. 1998), provided to our laboratory by Steven Reppert (University of Massachusetts, USA), further referred to as *Dm*PER 4Ep. The pCopia-*Renilla*

luciferase construct was a gift to our laboratory from Michael Rosbash (Brandeis University, USA).

Culture and transfection of S2 cells

Drosophila Schneider 2 cells (*S2* cells) (Invitrogen) were used as a convenient heterologous system for the luciferase assay. S2 originate from a primary culture of late stage (20 - 24 h old) *D. melanogaster* Oregon-R embryos (Schneider 1972) and do not express most circadian genes with the exception of *cyc* (McDonald and Rosbash 2001). S2 cells were cultured at 25°C in HyClone™ Insect cell culture media: SFX-Insect liquid medium with L-glutamine, sodium bicarbonate (GE Healthcare) with 10% heat-inactivated Fetal Bovine Serum (FBS; Gibco) and antibiotics (50 U Penicillin G with 50 ug/ µL Streptomycin - 1%); they were split every 3-4 days at dilution 1: 4.

Prior to transfection, S2 cells from 2-3 day old subcultures were diluted to $1x10^6$ cells/ mL and seeded overnight at 0.8 mL per well in 12-well plates. Cells were transfected transiently using 6 µL of Cellfectin® II Reagent (Invitrogen) following the manufacturer protocol and incubated for 5 h at 25°C. Each transfection was performed in triplicate with the same transfection mix, containing a total amount of plasmid DNA in a range of 260 ng - 380 ng. Control transfection contained 50 ng of *Dm*PER 4Ep, 30 ng of pCopia-*Renilla* and empty vector expression vector pAc5.1/V5-HisA. Each sample transfection consisted of 50 ng of *Dm*PER 4Ep, together with 30 ng of pCopia-*Renilla* and 50 ng of each pAc5.1-*Nvclk*, pAc5.1-*Nvcyc* (or pAc5.1-*Nvcyc*ΔC). To test the effect of other clock genes involved in the feedback loop on the transcriptional activity of *Nv*CLK:CYC I added pAc5.1-His6-*Nvcry2* (50 ng) or pAc5.1-*Nvper*-V5-His6 (50 ng) or both. Further, I expanded the transfection experiment in a dose dependent manner, including concentrations from 5 - 50 ng in case of pAc5.1-His6-*Nvcry2* and 50 - 100 ng for pAc5.1-*Nvper*-V5-His6. After removal of the transfection mixture, cells were incubated in media supplemented with antibiotics for 48 h at 25°C. Cells were then

washed with Dulbeccos phosphate-buffered saline (PBS; Gibco) and harvested and diluted in 1x Passive Lysis buffer (Promega). The protocols were adapted from Rosato (2007).

Luciferase assay

The luciferase assay was used to test the transcriptional regulation of *Nv*PER, *Nv*CLK, *Nv*CYC and *Nv*CRY2 in the clock feedback loop mechanism. The transcription activity of the *Nv*CLK:CYC dimer was assessed by measuring the expression of a reporter construct (pGL3 *4E-hsp-luc*) containing an E box, both in the presence and absence of *Nv*PER and *Nv*CRY2. Luciferase activity was measured using the Dual Luciferase Reporter Assay Kit (Promega) on a FLUOstar Omega (BMG Labtech) microplate reader, following the manufacturers instructions. The assay works with two reporters to increase accuracy of the experiment. The firefly luciferase under *Dmper* E-box promotor (Darlington et al. 1998), and an internal control to determine the baseline activity, *Renilla* luciferase under constitutive *Drosophila actin* promoter, were used as the two reporters (Rosato 2007). As expression of both reporters is measured, it allows normalizing the experimental expression to internal control. The assay reaction works on the bioluminescent reaction catalysed by firefly luciferase, which leads to its oxidation of substrate (luciferin) during which the light is released and can be quantified (Rosato 2007). Each of the luciferase is working with a different type of luciferin and therefore both can be measured in a single reaction. First the firefly luciferase activity is measured and after its reaction is stopped the second *Renilla* luciferase is simultaneously activated. For each sample the baseline luciferase activity was established with control transfection containing the reporter construct pGL3 4E-*hsp-luc*, pCopia-*Renilla* and the empty vector pAc5.1/V5-HisA. The firefly luciferase activity was normalised to *Renilla* luciferase activity.

RESULTS

Identification of putative clock genes

I identified the core clock genes of *Nasonia vitripennis* with WaspAtlas (Davies and Tauber 2015), including *period, Clock*, *cycle* and *cryptochrome 2,* through their homology with clock genes of other species. I also compared protein sequences of these genes with their orthologues from *Drosophila melanogaster*, *Apis mellifera* and *Mus musculus* to identify their functional domains and motifs.

CRYPTOCHROME2

Nasonia CRY2 (*Nv*CRY2) has sequence similarities in functional domains to "mammalian-type" CRY proteins (Bertossa et al. 2014 and this study Figure 2.2 and Supplementary table S1.1). It shares a highly conserved N-terminal region, also referred to as the Photolyase Homology Region (PHR), which contains a DNA-photolyase domain (DNA-PL) and a Flavin Adenine Dinucleotide binding region (FAD-B). PHR binds the cofactor FAD and the second light harvesting light antenna chromophore (Sancar 2003). A monopartite nuclear localization site (NLSn) within PHR is present in *Nasonia* CRY2, however the bipartite NLSc was not identified. Furthermore, the N-terminus has a Cytoplasmic Localization Domain (CLD), which is highly conserved within "mammalian-like" CRYs. Three Repressor Domains (RD-2a; RD-1;RD-2b), conserved in "mammalian-like" CRYs, are also present in *Nv*CRY2. The C-terminus of *Nv*CRY2 shares similar features with the "mammalian-like" C-terminus. The C-terminal region is variable in length and in amino acid sequence, resulting in functional differences (Sancar 2003; Partch and Sancar 2005; Mei and Dvornyk 2015). The Coil-coil region (C-C) preceding the C-terminal region is also present in "mammalian-like" CRY. *Nasonia* CRY2 contains domains and motifs important for its nuclear localization as well as sites necessary

for interaction with CLK:CYC, which are essential requirements to act as a transcriptional repressor.

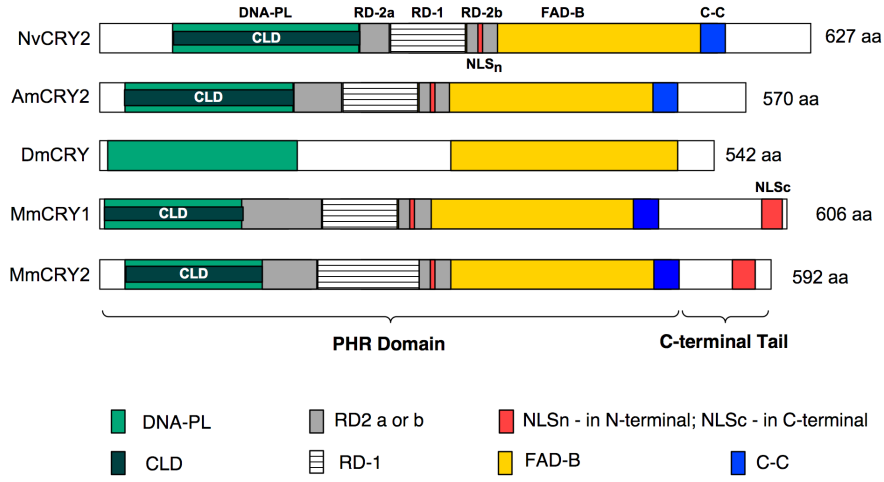

Figure 2.2. Schematic representation of CRY proteins from different species. Compared are *Nasonia vitripennis* CRY2 (*Nv*CRY2) with *Apis mellifera* CRY2 (*Am*CRY2), *Drosophila melagonaster* CRY (*Dm*CRY) and mouse CRY1 and 2 (*Mm*CRY1, 2) proteins with putative domains and motifs indicated by different colours. Polypeptide length in amino acids (aa) is shown on the right side. Abbreviations: PHR = Photolyase Homology Region; CLD = Cytoplasmic Localization Domain; NLSn = monopartite Nuclear Localization Signal in N-terminal region; NLSc = bipartite Nuclear Localization Signal in C-terminal region; RD = Repressor Domain; FAD-B = Flavin Adenine Dinucleotide Binding site; C-C = Coil-coil region.

PERIOD

Figure 2.3 shows a schematic representation of *Nasonia* PER protein (*Nv*PER) in comparison to PER orthologues from different species. *Nasonia* have sequence similarities in functional domains of PER-ARNT-SINGLEMINDED domain (PAS), PAC (PAS - associated C-terminal) and Period-C (the C-terminus of PER) (Figure 2.2 and Supplementary table S1.1). PAS has two tandem domains (PAS-A &PAS-B) through which they can dimerise. Furthermore, the PAC domain can act as a cytoplasmic localization domain (CLD) (Saez and Young 1996). *Nv*PER is more

similar to mammalian homologues, in particular regarding the presence of the Period C domain. Translocation into the nucleus could be facilitated via a putative nuclear localization signal (NLS), which was predicted within *Nv*PER (Figure 2.3) (Bertossa et al. 2014). NLS in PER was identified across all compared insect species. *Nasonia* is however missing the nuclear export signal (NES) motif.

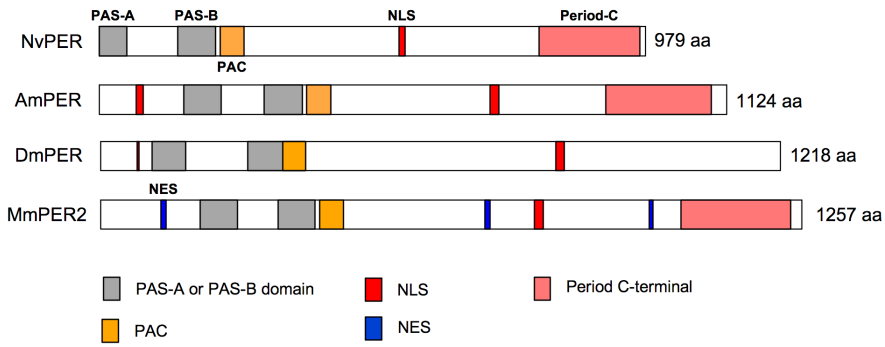

Figure 2.3. Schematic representation of PER protein from different species. Compared are *Nasonia vitripennis* PER (*Nv*PER) with *Apis mellifera* PER (*Am*PER), *Drosophila melagonaster* PER (*Dm*PER) and mammalian PER in mouse (homologue *Mm*PER2) proteins with putative domains and motifs indicated with different colours. Polypeptide length in amino acids (aa) is shown on the right side of the scheme. Abbreviations: PAS = PER-ARNT-SINGLEMINDED domain part A and B; PAC = PAS – associated C-terminal; Period-C (the C-terminal of PER); NLS = Nuclear Localization Signal; NES = Nuclear Export Signal.

CLOCK and CYCLE

Nasonia CLK and CYC proteins (*Nv*CLK, *Nv*CYC) include conserved domains of basic Helic-Loop-Helix (bHLH) motif, PAS-A, PAS-B and PAC domain (Figure 2.4 and 2.5). In the C-terminus of CLK contains Poly-glutamine repeats (polyQ), which show inter-species variability in length and abundance (Figure 2.4). *Nv*CLK possesses a shorter C-terminal region compared to *Drosophila*. The C-terminal region of *Nv*CYC is markedly longer than *Dm*CYC (Figure 2.5) and shows high similarity to the C-terminal region of BMAL1 (BCTR), which is highly conserved

among vertebrates and insect species, with the exception of *Drosophila*. Overall the structure of *Nv*CYC suggests that the transactivation domain is localized within *Nv*CYC rather than in *Nv*CLK.

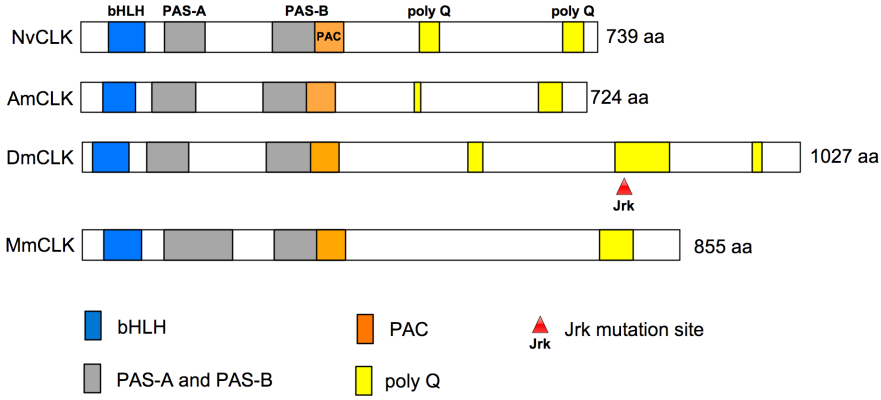

Figure 2.4. Schematic representation of CLK protein from different species. Compared are *Nasonia vitripennis* CLK (*Nv*CLK) with *Apis mellifera* CLK (*Am*CLK), *Drosophila melagonaster* CLK (*Dm*CLK) and mammalian CLK in mouse (*Mm*CLK) proteins with putative domains and motifs indicated with different colours. Polypeptide length in amino acids (aa) is shown on the right side of the scheme. Abbreviations: bHLH = basic Helic-Loop-Helix; PAS = PER-ARNT-SINGLEMINDED domain part A and B; PAC = PAS – associated C-terminal; poly Q = Poly-glutamine repeats.

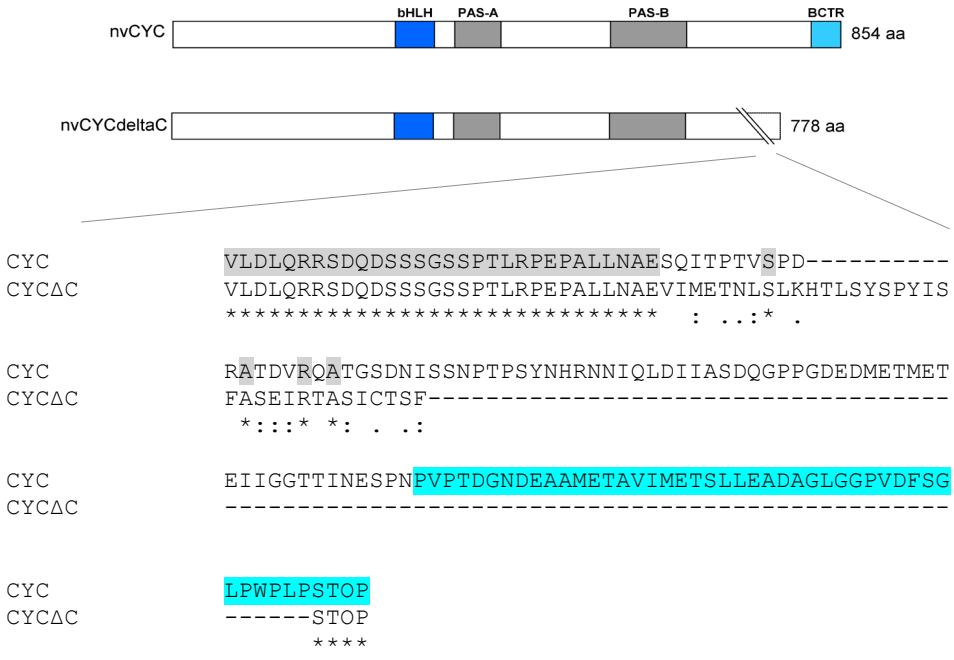

Figure 2.5. Schematic representation of *Nasonia* protein functional domains in CYC. Protein depicted with putative domains and motifs indicated with different colours. Lower bar is truncated version of CYC with highlighted changes under the scheme. Abbreviations: bHLH = basic Helic-Loop-Helix; PAS = PER-ARNT-SINGLEMINDED domain part A and B; BCTR = BMAL1 C-terminal Region.

Expression of circadian clock genes

Cyclical gene expression is a relevant characteristic of many clock and clock-controlled genes and can provide insight into the architecture of the clock. The cycling of *period* (*Nvper*), *Clock* (*NvClk*), *cycle* (*Nvcyc*), *cryptochrome2* (*Nvcry2*), *timeout* (*Nvtim2*) and *pigment dispersing factor* (*Nvpdf*) was examined in a 24 h time course experiment. Their expression was assessed by reverse-transcription, real-time, quantitative PCR (qPCR) using whole-head RNA extracts from males. Males were chosen as representatives for their stronger circadian rhythms. Under the LD 12:12 regime, samples were collected every 3 h (ZT1 - ZT22). Figure 2.6 shows a fit of a sinusoidal wave with 24 h periodicity upon forward linear harmonic regression for each gene expression data set. Putative *Nasonia* core clock genes did not show significant circadian oscillation with the exception of *Nvcyc* mRNA (ANOVA, *Nvper* ($p = 0.279$, $F = 1.38$, $r^2 = 0.38$); *NvClk* ($p = 0.598$, $F = 0.80$, $r^2 = 0.27$); *Nvcyc* ($p = 0.037$, $F = 2.89$, $r^2 = 0.56$); *Nvcry2* ($p = 0.264$, $F = 1.42$, $r^2 = 0.38$); *Nvtim2* ($p = 0.538$, $F = 0.89$, $r^2 = 0.29$); *Nvpdf* ($p = 0.694$, $F = 0.67$, $r^2 = 0.24$). *Nvcyc* has a peak of oscillation in the middle of the night and the lowest point in the first half of the light phase.

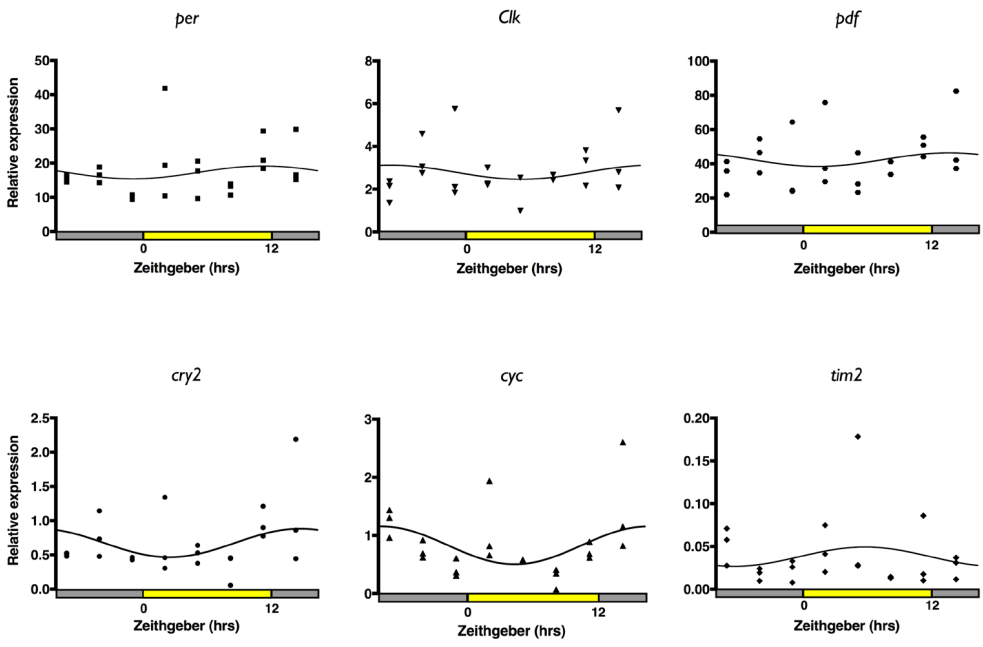

Figure 2.6. Expression level of *Nasonia* putative clock genes under a LD12:12 cycle. Relative mRNA abundance in males whole head extracts, over 24 h during light (yellow bar) and dark (grey bar) phases. Wasps were entrained to LD 12:12 and samples (40-50 individuals) were collected every 3 h for a full cycle. For each gene expression data set the line represent the fit of a sinusoidal curve with 24 h periodicity applying a linear harmonic regression; n = 2-3 biological replicates per time point.

Gene expression levels at CT3 and CT15 under LL conditions are shown in figure 2.7. *Nvper* had significantly higher expression at CT3 than CT15 ($p < 0.0001$). *Nvcry2* showed a significantly opposite expression pattern to *Nvper* ($p < 0.0001$). However, it is important to note that results for *Nvcry2* are derived from a single biological replicate, as the other biological replicates were not used, due to substantial variation between technical replicates (more than 0.5 cycle). Lastly, *NvClk*, *Nvcyc* and *Nvpdf* showed no significant difference in expression between CT3 and CT15 in LL.

Nvtim2 had significantly higher levels of expression at CT3 than CT15 ($p < 0.0001$). Of the genes showing circadian oscillation in a recent RNAseq study (Davies and Tauber 2016), *Nvopsin LW*, *NvLOC 464* and *Nvp450* had significantly

higher mRNA levels at CT15 compared to CT3 ($p < 0.0001$, $p < 0.05$ and $p < 0.0001$ respectively). *Nvallst* showed no significant difference between the two time points. The results indicate different gene expression profiles of putative core clock genes under LD and LL conditions. Under LD only *Nvcyc* shows circadian oscillation, whereas in the LL regime *Nvper*, *Nvcry2* and *Nvtim2* oscillate.

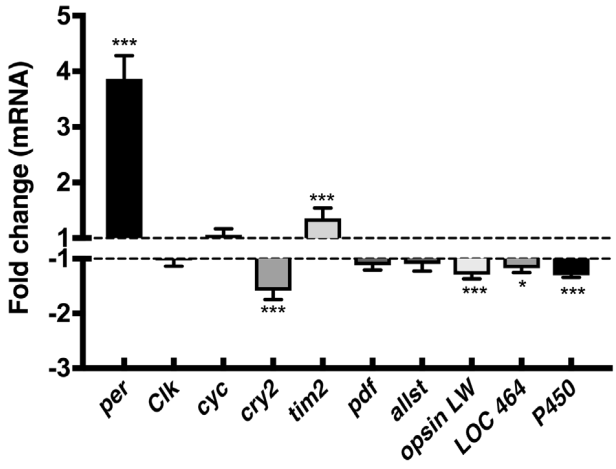

Figure 2.7. Comparison of gene expression at CT3 and CT15. Dash lines represent fold-change of 1 (no difference between groups), above 1 = mRNA levels at CT3 > CT15, below -1 = CT3 < CT15. Mean ± SD; statistically evaluated with permutation; *** $p < 0.0001$, * $p < 0.05$; n = 5 per gene and time point with exception of *Nvcry2* at CT3, n = 1.

Luciferase Reporter Assay

Luciferase reporter assay allows functional testing of a single clock gene for its transcriptional activity. Cells were first transfected with *NvClk* to test for possible interactions with endogenously expressed *cyc* (Darlington et al. 1998), which would complicate independent testing of the *Nasonia* clock system. No luciferase activity was detected after transfection with *Nv*Clk only. This means that endogenously expressed *cyc* does not interact with *Nv*Clk to activate the reporter from the *Dmper* promoter (Figure 2.8). Next, *Nvcyc* was co-transfected to see if it would create a

heterodimer with *NvClk* and activate the transcription of the reporter from the *Dmper* promoter. The addition of *Nvcyc* led to a notable but not significant transactivation of *Dmper 4Ep* (ANOVA: $F_{5, 12} = 4.12$; Tukeys *post hoc* test $p = 0.12$) (Figure 2.8). Based on sequence similarities of the BMAL/CYC family of proteins with *Nv*CYC (Supplementary table S1.1), I further tested the role of *Nv*CYC C-terminal region for its transactivation function (Chang et al. 2003). The BMAL C-terminal region (BCTR) is known for its strong *in vitro* transcriptional activity in mammals and shows high similarity among insect species, including *Nasonia*, but not *Drosophila* (Takahata et al. 2000; Chang et al. 2003). I generated a truncated version of *Nv*CYC with a deletion in the C-terminal region (including BCTR), referred to as *Nv*CYCΔC (Figure 2.5). Adding *Nvcyc*ΔC to *NvClk* yielded only baseline activity of *Dmper 4Ep* reporter, thus confirming the localization of the transactivation domain within the C-terminal region of *Nv*CYC (Figure 2.8). The addition of *Nvper* together with *NvClk* and *Nvcyc* did not inhibit the activity of the reporter, suggesting that *Nv*PER on its own does not inhibit *Nv*CLK:CYC transcriptional activity. On the other hand, the addition of *Nvcry2* to *NvClk* and *Nvcyc* led to a notable but not significant decrease of reporter activity (ANOVA: $F_{5,12} = 4.12$; Tukeys *post hoc* test $p = 0.1$).

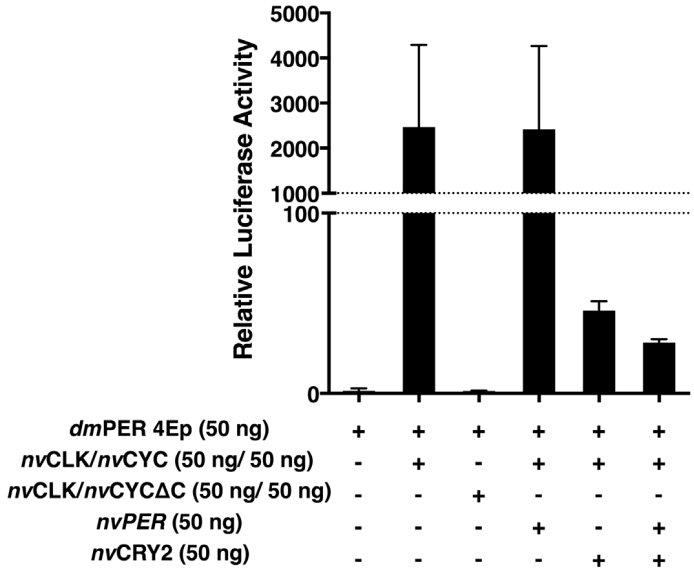

Figure 2.8. Luciferase Gene Reporter Assay in *Drosophila* S2 cells testing transcriptional activity of putative *Nasonia* clock genes. *Drosophila per* E-box luciferase reporter (*Dm*PER 4Ep) was used in presence (+) or absence (-) of *Nv*CLK/CYC (or *Nv*CYCΔC), *Nv*CRY2 and *Nv*PER (mass in ng indicated in brackets). Luciferase activity was calculated relative to *Renilla* Luciferase. Each value is mean ± SD with independent transfections; ANOVA; Tukeys *post hoc* test; n = 3.

A dose dependent analysis further confirmed the inhibitory effect of *Nv*CRY2 on *Nv*CLK/CYC transcriptional activity (ANOVA: $F_{5,11} = 4.62$; Tukeys *post hoc* test (5 ng) $p > 0.05$; (15 ng; 30 ng; 50 ng) $p < 0.05$) and the lack of repression exerted by *Nv*PER alone (ANOVA: $F_{8,18} = 245.5$; Tukeys *post hoc* test (50 ng; 100 ng) $p > 0.05$) (Figure 2.9). The higher amount of *Nv*PER (250 ng) leads to increase in *Nv*CLK/CYC transcriptional activity ($p < 0.0002$). On the other hand, I observed positive interaction between *Nv*PER and *Nv*CRY2, as *Nv*PER seems to have tendency to enhance the inhibitory effect of *Nv*CRY2 (Figure 2.9).

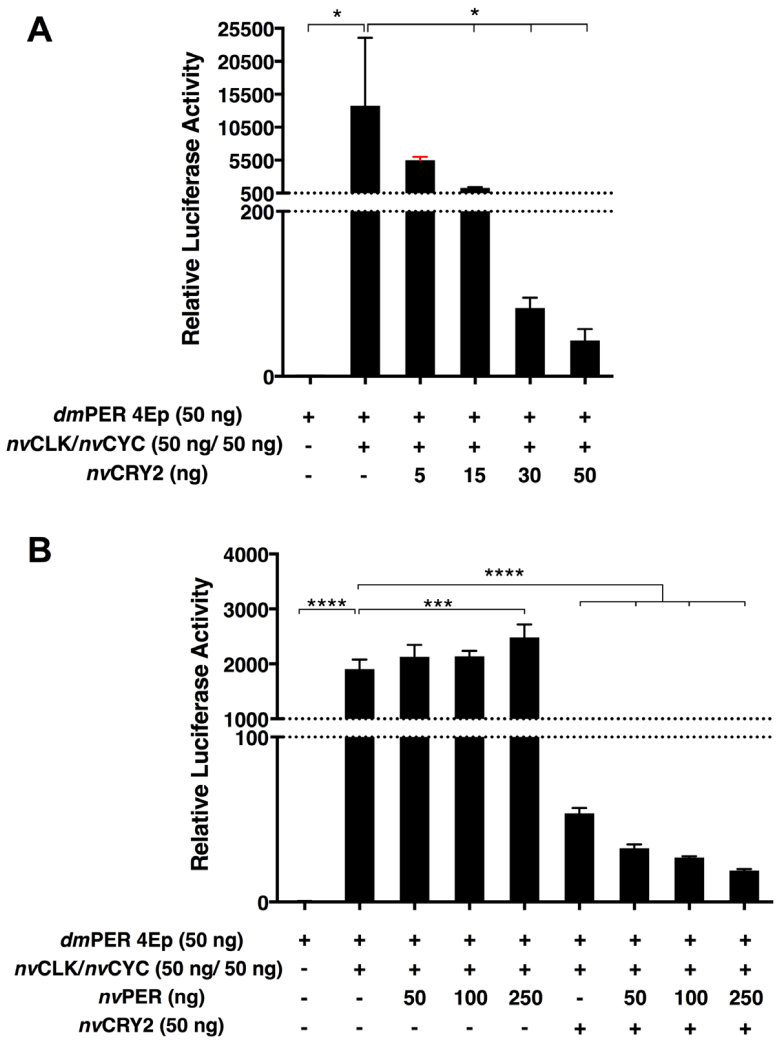

Figure 2.9. Dose dependent transcriptional response in the Luciferase Reporter Assay. The *Drosophila per* E-box luciferase reporter (*Dm*PER 4Ep) was transfected in the presence (+) or absence (-) of *Nv*CLK/CYC, *Nv*CRY2 and *Nv*PER (mass in ng indicated in brackets). Firefly luciferase activity was calculated relative to that of *Renilla* Luciferase. Each value is mean ± SD with independent transfections, ANOVA; Tukeys *post hoc* test; n = 3 (black error bar), n =2 (red error bar).

DISCUSSION

The current study provides a functional analysis of clock genes in the emerging model organism *Nasonia vitripennis.* Hymenopteran species show differences in clock organisation (Rubin et al. 2006) compared to other insect species, such as *Drosophila*. *Nasonia* was found to share more sequence similarity in functional domains of clock gene products with mammals than with *Drosophila,* consistent with results from the honeybee (Rubin et al. 2006). Like other Hymenoptera*, Nasonia* lacks "*Drosophila*-like" CRY and TIM, but possesses the "mammalian-like" CRY2 and TIM2 (Yuan et al. 2007; Zhan et al. 2011). CRY2 is very similar in sequence to "mammalian-like" CRY in agreement with Bertossa et al. (2014). Mammalian CRYs contain a N-terminal chromophore binding PHR domain that in mammals is important for interaction with the CLK:BMAL1 heterodimer (Chaves et al. 2006). Three repressor domains (RD-2a; RD-1; RD-2b), conserved in "mammalian-like" CRYs, have various functions (Hirayama et al. 2003). RD-1 and RD-2b are necessary for the interaction with CLK:BMAL1 heterodimer and therefore mCRY inhibitory functions; RD-2b and also RD-1 are involved in nuclear localization as it includes functional NLS (Hirayama et al. 2003; Hirayama and Sassone-Corsi 2005; Chaves et al. 2006). In mammals, a C-C region is involved in binding to BMAL1, but also to PER and therefore regulates stability and cellular localization of PER proteins (Chaves et al. 2006). The lack of similarity with *Drosophila* CRY in the C-terminal region makes it less likely that *Nasonia* CRY2 is light sensitive, as the C-terminal region is involved in light response in *Drosophila* (Rosato et al. 2001; Busza et al. 2004; Dissel et al. 2004).

Another difference between *Nasonia* and *Drosophila* is the sequence of the transactivation domain of CLK. *Nv*CLK does not have similarities in the polyQ rich C-terminal region of *Drosophila* CLK known for its transactivation function (Allada et al. 1998). Instead, a similar region to the transactivation domain of the BMAL C-terminal region (BCTR) is present in *Nv*CYC as shown by our dysfunctional *Nv*CYCΔC in the luciferase assay. The potent transcriptional activity

of the BCTR has also been shown previously *in vitro* studies using mammalian and lepidopteran BMAL1/CYC proteins (Takahata et al. 2000; Chang et al. 2003).

The gene expression pattern can be used to predict the architecture of the feedback loop mechanism that forms the molecular basis of the clock. Usually, genes that are transcribed and translated together show similar expression pattern, as demonstrated in several insect studies (Sauman and Reppert 1996; Goto and Denlinger 2002; Iwai et al. 2006). On the other hand, transcription factors exhibit an almost opposite phase to genes which they regulate (Sauman and Reppert 1996; Goto and Denlinger 2002; Iwai et al. 2006). Expression patterns of the *Nasonia* canonical clock genes in LD 12:12 regime is similar to *Apis* and mammals and different from *Drosophila* (Glossop et al. 2003; Rubin et al. 2006). This was evident from the expression patterns of *NvClk* and *Nvcyc*. *NvClk* is expressed continuously during 24 hours, but *Nvcyc* is rhythmically expressed with a peak in the middle of the night. Constitutive *Clk* expression, but rhythmic *cyc* expression, was also observed in several other insects, such as honeybee, cricket, pea aphid and firebrat (Rubin et al. 2006; Moriyama et al. 2008; Cortés et al. 2010; Kamae et al. 2010). The clock genes *Nvper, Nvcry2, Nvtim2* or *Nvpdf* did not show significant circadian oscillation, which might be due to the large variation between biological replicates. An increase in the number of biological replicates might be required to confirm the indication of an oscillatory pattern in those genes.

The expression patterns of the *Nasonia* clock genes were also investigated under LL conditions. I compared mRNA levels from CT3 and CT15 of the core clock genes together with other genes that were previously shown to undergo circadian oscillation (Davies and Tauber 2016) - *allatostatin* (*Nvallst*), *long wave opsin* (*Nvopsin LW*), gene *loci 464* (*NvLOC 464*) and *p450* (*Nvp450*). A constant light regime usually results in arrhytmicity in *Drosophila* (Emery et al. 2000), and alters free running period, eventually leading to arrhytmicity in mice (McMahon et al. 2005). However, it does not affect *Nasonia*, which keeps locomotor activity rhythmic under LL as well as in DD (Bertossa et al. 2013). Microarray and RNAseq data from mosquitoes, bees and also *Nasonia*, provided information about rhythmic

expression of clock genes and clock-controlled genes in constant light conditions (either LL or DD) (Rodriguez-Zas et al. 2012; Rund et al. 2011; Davies and Tauber 2016). My data from LL qPCR experiments revealed differences in expression levels of several putative clock genes and CCGs. *Nvper* has almost 4 times higher expression at CT3 than at CT15, which is in contrast to the LD results, where *Nvper* does not show a significant rhythmic expression. RNAseq time-course over 48 h (Davies and Tauber 2016) did not reveal rhythmic expression of *Nvper* under LL. On the other hand, *Nasonia* females show significant oscillation of *Nvper* and *Nvcry2* in LL and DD after being entrained in 12:12 LD (Bertossa et al. 2014), which is similar to bees in DD (Rubin et al. 2006).

No significant changes in expression levels were found for *Nvcyc* at CT3 compared to CT15. Davies and Tauber (2016) found significant circadian oscillation in *Nvcyc* gene expression in LL using RNA-seq. Significant cycling in mRNA of *Amcyc* was also found in bees under DD, but not in *AmClk* (Rubin et al. 2006). My qPCR data did not show significant oscillation of *NvClk* mRNA under LD or changes in expression at CT3 and CT15 in LL regime either. Again, different results were obtained from RNA-seq data by Davies and Tauber (2016) who found rhythmic expression under DD, as well as LL. These discrepancies between qPCR and RNA-seq data could be caused by several factors. First, I have analysed just two time points and therefore might have missed the peak amplitude cycling, caused by a shift from LD to LL. Second, the amplitude of the rhythm could be weaker under the free running conditions than in LD. Lastly, there may be experimental error in measuring circadian gene expression between biological replicates in qPCR, or because of low number of replicates used in the RNA-seq study.

I found that *Nv*CRY2 is the main inhibitor of *Nv*CLK:CYC mediated transcription in *Nasonia*. This was demonstrated with *Drosophila* S2 cells, where the co-transfection of *NvClk* and *Nvcyc* with *Nvcry2* caused reduction in transcription from the *Drosophila* E-box luciferase reporter. A similar finding was obtained from the monarch butterfly, using *Dp*N1 cells (*Danaus plexippus* cell line)

(Zhu et al. 2008), and further confirmed by *in vivo* knockout of CRY2 by zinc-finger nuclease (ZFN) induced mutation (Merlin et al. 2013). In a comparative study by Yuan et al. (2007) of the two types of insect CRY, substantial evidence was provided that CRY2 (from *Anopheles*, *Antheraea*, *Apis*, *Danaus* and *Tribolium*) acts similarly to "mammalian-like" CRY (mCRY1) as a potent transcriptional inhibitor within a *Drosophila* S2 cell model. On the other hand, CRY1 of *Anthearea*, *Danaus* or *Drosophila* is not sufficient to repress activity of CLK:CYC mediated transcription (Zhu et al. 2005; Yuan et al. 2007).

My study provides evidence that *Nv*PER is unable to repress *Nv*CLK:CYC in S2 cells, hence it is likely that it is unable to regulate its own transcription *in vivo*. This observation fits the model of Yuan at al. (2007) for species lacking CRY1. My dose response experiments revealed that *Nv*PER in combination with *Nv*CRY2 provides only a slight increase in the inhibition of *Nv*CLK:CYC transcriptional activity. Hence, the role of *Nv*PER in the *Nasonia* clock is not clear yet. Possibly, it has a role in the regulation of CRY2, similar to the mammalian counterparts where it regulates CRY2 nuclear translocation (Reppert and Weaver 2001) or similarly to butterflies to stabilize CRY2 (Zhu et al. 2008).

With the luciferase assay I also demonstrated that *Nv*CYC is essential for transcriptional activity of *Nv*CLK:CYC. The *Nv*CYC C-terminal region possesses a BCTR region similar to mammals and other insect species, but not *Drosophila.* A truncated version of *Nv*CYC (*Nv*CYCΔC), in which the BCTR region is missing, is unable to form a functional dimer with *Nv*CLK as suggested by the lack of reporter expression in S2 cells. This suggests that the transactivation domain of the complex is located in the BCTR region of *Nv*CYC and not in CLK as it is in *Drosophila* (Takahata et al. 2000; Chang et al. 2003; Uryu et al. 2013).

To conclude, the circadian clock of *Nasonia* seems to be more similar to the mammalian than to the *Drosophila* clock. The results of this chapter concerning the composition and regulation of genes are consistent with the proposed model for hymenopteran species by Yuan et al. (2007). The picture now looks as follows. The Nasonia clock consists of an autoregulatory transcriptional-translational

feedback loop in which *Nv*CLK:CYC heterodimers drive the transcription of *Nv*PER and presumably *Nv*CRY2 through E-box enhancer elements, CACGTG, found within 5 flanking region of *Nvcry2* and *Nvper*. Transcriptional activity is facilitated through the BCTR region on *Nv*CYC. *Nv*CRY2 is translocated to the nucleus to inhibit *Nv*CLK:CYC mediated transcription either alone or in complex with other proteins (Zhu et al. 2005). Whether *Nv*PER could stabilize *Nv*CRY2 before entering the nucleus, as in *Danaus* (Zhu et al. 2008), or help to translocate *Nv*CRY2 in the nucleus, if translocated to nucleus, as in mouse (Reppert and Weaver 2002), is still an open question. Moreover, the light pathway for circadian entrainment in *Nasonia* is not yet known. CRY2 is a potential candidate as it may have a dual role, not only as a transcriptional inhibitor, but also a photoreceptor (Rubin et al. 2006). The analysis of CRY2 functional domains performed in this chapter suggests that this is unlikely, however the possibility is investigated further in the next chapter.

ACKNOWLEDGEMENTS

I would like to thank Ane Martin Anduaga for introduction into cloning. Thanks belong also to Laura Delfino for Gibson assembly explanation and providing me with the firs sets of reagents for it. Ben Hunt and Giorgio Fedele for work with S2 cells and Luciferase Assay, especially for all help with troubleshooting. I would like to thank Eran for all his suggestions and Ezio Rossato for his comments on this chapter. Lastly, thank you all members of INsecTIME for your suggestions and comments.

APENDICES 1

Supplementary table S1.1. *Nasonia* putative circadian protein structure similarity (Query cover/Coverage), Identity in % and length in amino acids (AA) of putative domains of *Nasonia,* along with other species

CRYPTOCHROME

Species	Protein ID	Query cover (%)	Identity (%)	Lenht (AA)	Conserve Domains							
					DNA photolyase		FAD_binding_7		RD-2A		RD-2B	
Nasonia vitripennis	XP_001606405	-	-	627	Coverage (%)	Identity (%)	Coverage (%)	Identity (%)	Coverage (%)	Identity (%)	Coverage (%)	Identity (%)
Apis mellifera	NP_001077099	82	88	570	98	87	100	94	89	44	100	82
Drosophila melanogaster	NP_732407	77	40	542	99	42	99	43	75	83	35	50
Mus musculus	NP_031797	83	71	606	98	54	100	80	95	59	100	73
Mus musculus	NP_034093	82	68	592	100	64	100	78	91	62	100	73

CRYPTOCHROME

Species	Protein ID				
		RD 1		Coiled coil	
Nasonia vitripennis	XP_001606405	Coverage (%)	Identity (%)	Coverage (%)	Identity (%)
Apis mellifera	NP_001077099	100	96	100	87
Drosophila melanogaster	NP_732407	94	39	-	-
Mus musculus	NP_031797	100	78	95	68
Mus musculus	NP_034093	100	78	95	68

PERIOD

Species	Protein ID	Query cover (%)	Identity (%)	Lenht (AA)	Conserve Domains							
					PAS-A		PAS-B		PAC		Period C	
Nasonia vitripennis	XP_008209247	-	-	979	Coverage (%)	Identity (%)	Coverage (%)	Identity (%)	Coverage (%)	Identity (%)	Coverage (%)	Identity (%)
Apis mellifera	NP_001011596.1	97	45	1124	96	53	100	83	100	75	84	38
Drosophila melanogaster	NP_525056.2	68	40	1218	96	51	95	49	100	73	-	-
Mus musculus	NP_035196.2	40	31	1257	78	29	100	38	95	52	46	29

CYCLE

Species	Protein ID	Query cover (%)	Identity (%)	Lenht (AA)	Conserve Domains							
					bHLH		PAS-A		PAS-B		BCTR	
Nasonia vitripennis	XP_008215805.1	-	-	854	Coverage (%)	Identity (%)	Coverage (%)	Identity (%)	Coverage (%)	Identity (%)	Coverage (%)	Identity (%)
Apis mellifera	XP_016770595.1	74	77	675	100	98.039	94.915	98.31	94.79	85.417	100	97.436
Drosophila melanogaster	NP_524168.2	47	60	413	94.12	88.235	80	90	84.38	63.542	0	0
Mus musculus	NP_031515.1	66	50	626	93.02	74.419	74.576	91.53	77.89	62.105	96.88	96.875

CLOCK

Species	Protein ID	Query cover (%)	Identity (%)	Lenht (AA)	Conserve Domains							
					bHLH		PAS-A		PAS-B		PAC	
Nasonia vitripennis	XP_008214438.1	-	-	739	Coverage (%)	Identity (%)	Coverage (%)	Identity (%)	Coverage (%)	Identity (%)	Coverage (%)	Identity (%)
Apis mellifera	XP_394233.4	46	80	724	100	92	98	78	98	95	100	93
Drosophila melanogaster	NP_001014576.1	70	53	1027	96	78	91	61	100	77	100	71
Mus musculus	AAC53200.1	51	49	855	98	67	91	43	98	83	100	86

Supplementary table S1.2. Primer pairs used for qPCR, cloning and Gibson Assembly

Primer		Primer sequence (5' -> 3')	
Gene	Name	Forward	Reverse
qPCR			
Clock	*nv*_clock_a	TGAAAGTAGAGCAACAGCATTGT	TGACGCATCTCGGTCGTTTG
cryptochrome 2	*nv*_cry2_5/6_b	GGCATTACCGTCGTCCAGAG	AAGTCATCGTGGTCGTCCTT
cycle	*nv*_cycle_d	GGTTCGACATCCTCCATCCC	CCTGAGGCATGTCCGTCTTT
period	*nv*_per_a	TCTCGTCGCCTTCTTCCAAC	ATCGGGATCGACGTAGGACA
pdf	*nv*_pdf_a	TTCGTTTTCGGAGCCATCCT	TCAGTTCGGCATCCAATGAAC
allatostatin	*nv*_allst_2	CTGGGTCGACGATAAGCGAT	TAGCTCAGATCCAGGCCGTA
LOC 464	*nv*_LOC464_1	GCAGGGCTACAAAGGTGAGA	CTGTAGAACACGGGAACGCA
opsin LW	*nv*_opsinLW_2	TGGCTGGAACCGGTACGTC	TCATGAACAGGGGCAGGAAG
p 450	*nv*_p450	CGCTGTTTTCAACGAGACCC	TAACGGGTTTACCGCCTGAC
rpl32	*nv*_rpl32_nv	GCCCAACATCGGTTATGGT	AACTCCATGGGCAATTTCTG
timeout	*nv*_tim2_a	GCAAGCGAGGTTCCCATAGA	CAGACTTCCCTTGCCGAGC
Cloning			
cryptochrome 2	cry2_500_F	CAATCACGAGAACACTATCTACTTC	ATCGCGTATGTTCAGTTTCTTG
period	per_clon_F3_301	CTCACTGCGCTACCTCAACA	ATGGTCGGTAATTGTCCACGG
Clock	clock_483_F2	TGCCAGTATAGTGCCTCCCT	TCTCTAATTTCGGGTTGATCTATGT
cycle	cycle_585_F2	TCATCATCATCAGAGCCACGG	CTACGAAGGGCAACCCGAAA

Primers for Gibson Assembly

Gene		Primer sequence (5' -> 3')
cryptochrome 2	Forward	acagtggcggccgctcgagtctagacaaagccatgcaccaccaccaccaccacggcggaggtggttcaATGACGGGTAGCCAGGGC
	Reverse	aggcttaccttcgaagggcccTTAATGATATTGATGTTGATGAACGTG
period	Forward	acagtggcggccgctcgagtctagacaaagccATGCATGACGGCCTTGTG
	Reverse	taccttcgaagggcccaaAAGAGTGCTCCACGATTATTTC
Clock	Forward	tccagcacagtggcggccgctcgagcaaagccATGGCGAGTTGTATTAGAAAC
	Reverse	gcttaccttcgaagggccctctagaTTAAAGTAGGTCGTTCTGTTG
cycle	Forward	acagtggcggccgctcgagtctagacaaagccATGGTCGTCCAGGAGAGTC
	Reverse	aggcttaccttcgaagggcccTCAAAAAGATGTACATATACTTGCCG

CHAPTER 3

Nasonia CRY2 is involved in circadian behaviour regulation but not photoreception

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ABSTRACT

The parasitoid *Nasonia vitripennis* shows clear light-dependent circadian and seasonal rhythms, the paces of which are dictated by an internal clock(s). However, unlike some other insects, Hymenoptera do not possess the light sensitive clock component CRYPTOCHROME 1 (CRY1), but instead a mammalian type of CRYPTOCHROME, CRY2. How light is processed by the clock in the absence of CRY1, and whether CRY2 plays this role in hymenopterans is unknown. Here, I investigate a possible dual role of CRY2 as a photoreceptor and transcriptional repressor in *Nasonia*. I measure locomotor activity under various light conditions and after applying a 1-hour light pulse during the dark phase. The obtained phase response curves (PRC) are similar for males and females with peak sensitivities at Zeitgeber time ZT14 and ZT22. *Nvcry2* RNAi knockdown results in a significant attenuation of phase advance after a light pulse at ZT23 in males, but not females. However, knockdown of *Nvcry2* did not affect the ability of males to re-entrain to a phase advanced light-dark cycle under a range of different wavelengths. Exposure to light did not affect the function of *Nv*CRY2 as a transcriptional repressor in the S2 cell luciferase transcription assay. Together, these results indicate that *Nasonia cry2* is a circadian clock gene, but does not function as a photoreceptor in circadian entrainment. Jet-lag assays under different light wavelengths revealed that wasps re-entrain faster to long (> 516 nm) wavelengths than short (< 464 nm). Alternative, most likely long wavelength photoreceptor(s) thus appear necessary for circadian light-entrainment in this species.

INTRODUCTION

The most reliable environmental cue for circadian and photoperiodic entrainment is light (Yoshii et al. 2016). Light information is relayed through to the circadian clock of insects via several visual or non-visual photo-transduction pathways. The visual pathways include the ocular (compound eyes and ocelli) and the extraocular photoreception (Hofbauer-Buchner eyelets). The non-visual pathways involve light input factors such as CRYPTOCHROME (CRY), QUASIMODO, presumably PTEROPSIN and the recently characterized rhodopsin - Rh7 (Stanewsky et al. 1998; Velarde et al. 2005; Chen et al. 2011; Yoshii et al. 2015; Ni et al. 2017). QUASIMODO is a membrane-anchored extracellular protein. It affects the light response of clock neurons, possibly by upstream regulation of several ion channels, as determined in *Drosophila* (Buhl et al. 2016). PTEROPSIN is a non-visual vertebrate-like opsin found in honey bees, *Apis mellifera* (Velarde et al. 2005). Like Pigment Dispersing Factor (PDF), it is expressed in so-called pacemaker neurons in the optic lobe (Bloch et al. 2003). PTEROPSIN might be involved in light regulation of the clock in bees, as its expression pattern corresponds to regions of the brain often associated with clock coordination in insects (Helfrich-Förster et al. 1998; Bloch et al. 2003). Rh7 contributes to the light entrainment of pacemaker neurons via violet light in *Drosophila* (Ni et al. 2017). CRYPTOCHROME (CRY), on the other hand, is a blue light receptor (Stanewsky et al. 1998), expressed together with Rh7 in PDF positive neurons in *Drosophila* (Ni et al. 2017). Thus, there are various pathways of light entrainment in insects, each depending on different photoreceptors, and their precise regulation may differ between species.

Besides the type of CRY described in *Drosophila* (further referred to as CRY1), a CRY homologue was identified, first in butterflies and later in other insect species (Zhu et al. 2005; Yuan et al. 2007). This second type of CRY, more similar to “mammalian-like” CRY is further referred to as CRY2. Insects can be divided

into several groups based on the presence or absence of CRY homologues (details in Chapter 2). Drosophilid species possess only CRY1, whereas other insects carry only CRY2 or both variants (Table 3.1) (Yuan et al. 2007).

CRY1 is involved in circadian response to light in *Drosophila*, the monarch butterfly *Danaus plexipus*, the mosquito *Anopheles gambiae*, and presumably other insects that carry CRY1 (Emery et al. 2000; Yuan et al. 2007; Zhu et al. 2008). In contrast to CRY1 (Rosato et al. 2001; Peschel et al. 2009), CRY2 appears to be light insensitive (Yuan et al. 2007). However, there are several studies reporting "mammalian-like" CRY photic responsiveness. For example, human mCRY1 (mammalian CRY exists in two paralogues named mCRY1 and mCRY2) expressed in Sf21 insect cells and *Drosophila in vivo*, undergoes photoreduction after exposure to blue light (Hoang et al. 2008). Also, *Drosophila cry*-null mutants carrying human mCRY2 are able to show light-dependent response to an electromagnetic field in blue light (Fedele et al. 2014). The light-dependent magnetoreception of insect CRY2 was also shown in cockroaches *in vivo* (Bazalova et al. 2016). Hence, in insects, both CRY1 and CRY2 may be light sensitive depending on which species is being considered, but a comprehensive picture of the role of these two genes in insect photoreception is still missing.

Table 3.1. Overview of the presence of canonical clock genes in insects.

✓ marks presence of the clock gene, ✗ absence or **?** missing information (adapted from Zhang et al. 2010 and Tomioka and Matsumoto 2015).

Species	Clock gene						
	per	***tim***	***timeout***	***clock***	***cyc***	***cry1***	***cry2***
Diptera							
Drosophila melanogaster	✓	✓	✓	✓	✓	✓	✗
Musca domestica	✓	✓	?	✓	✓	✓	✗
Aedes aegypti	✓	✓	✓	✓	✓	✓	✓
Anopheles gambiae	✓	✓	✓	✓	✓	✓	✓
Lepidoptera							
Antheraea pernyi	✓	✓	?	✓	✓	✓	✓
Bombyx mori	✓	✓	✓	✓	✓	✓	✓
Danaus plexippus	✓	✓	✓	✓	✓	✓	✓
Hymenoptera							
Solenopsis invicta	✓	✗	✓	✓	✓	✗	✓
Nasonia vitripennis	✓	✗	✓	✓	✓	✗	✓
Apis mellifera	✓	✗	✓	✓	✓	✗	✓
Coleoptera							
Tribolium castaneum	✓	✓	✓	✓	✓	✗	✓
Hemiptera							
Acyrthosiphon pisum	✓	✓	✓	✓	✓	✓	✓
Riptortus pedestris	✓	?	?	✓	✓	?	✓
Orthoptera							
Gryllus bimaculatus	✓	✓	?	✓	✓	✓	✓
Rhyparobia maderae	✓	✓	?	?	?	?	✓
Thysanura							
Thermobia domestica	?	✓	?	✓	✓	?	✓

Both visual pathways as well as CRY1 are required in *Drosophila* for light entrainment. However CRY1 is the main photoreceptor responsible for *Drosophilas* ability to phase shift the clock (Stanewsky et al. 1998; Schlichting et al. 2015). CRY1 acts through an interaction with TIMELESS (TIM) in a light dependent manner, which leads to TIM and CRY proteasomal degradation (Lin et al. 2001). In insect species that do not have CRY1 but only CRY2, an alternative photoreceptive pathway to synchronize the clock with the light presumably exists. Such a pathway could be directed through TIM degradation via a CRY-independent pathway (Yuan et al. 2007). Interestingly, in Hymenoptera (including the wasp *Nasonia vitripennis*) not only CRY1 is missing, but also TIM, which opens the possibility for yet another light synchronisation mechanism. Here, I address the question whether *Nv*CRY2, given the evidence for its light sensitivity, could play a role as a photoreceptor in *Nasonia*, besides its role as negative regulator in the core clock (Chapter 2).

MATERIALS AND METHODS

Wasp strains and rearing conditions

The AsymCx strain of the parasitic wasp *Nasonia vitripennis* was used in all experiments. This strain has been cured from *Wolbachia* infection, originates from The Netherlands (van den Assem and Jachmann 1999) and has been used for the genome project (Werren et al 2010). It is maintained under a light-dark cycle of LD 12:12 at 25 °C in glass or plastic vials (70x20 mm). Pupae of *Calliphora* flies are used as hosts. The mass cultured laboratory stock is maintained on a 14-day cycle by providing approximately 10-20 females with 30-50 fly hosts each generation.

Behavioural assays

Free running rhythms

Locomotor activity behaviour was measured to assess the circadian response

across different experimental conditions and treatments. The Drosophila Activity Monitoring (DAM) system (Trikinetics, USA) was used to measure locomotor activity. Individual newly emerged (24-48 h of age) wasps were placed into glass tubes and closed with sucrose agarose gel at one end and a clean cotton plug at the other. The monitors were kept in light boxes equipped with white LEDs (intensity 150-250 lux), in an incubator maintained at 18.5 ± 1°C. Animals were entrained for three days in LD 12:12 cycles, followed by constant light (LL) or darkness (DD) for at least 5 days.

In separate set of experiments, the free running period of the wasps was measured in dim blue light constant conditions. In these experiments the wasps were entrained for 2-3 days in LD 12:12 in white LED light of intensity 0.25 $\mu W/cm^2$, followed by constant blue light (464 nm) of the same intensity.

The free running period (τ) was calculated using a custom made software (BeFly!, developed by Ed Green), with cosine algorithm implemented from (Refinetti et al. 2007). Four days in LL (or DD) were used to calculate the period (τ) (omitting the first day of constant conditions as a transition day) as well as the rhythm strength (F) as a determination of the best fit of the cosine wave. Only rhythmic animals were used to calculate τ. Rhythmic individuals were defined as those whose calculated maximal F-value from cosine wave fit was over 99% confidence interval. Robustness of the rhythm was estimated as the percentage of the variance based on the fit of the cosine curve for a given period.

Phase response curve

A phase response curve (PRC) was used to obtain information about the endogenous clock response to light stimulus during the subjective dark phase. This information was also used to compare the response of dsRNA treated wasps (see below) with that of the wild type control. PRC was created by series of experiments in which a short light stimulus was delivered during constant darkness (Daan and Aschoff 2001). The light pulse was delivered at different time points during the

night. The anchor protocol was employed, where the light pulse was delivered during the last night of entrainment. This allow the endogenous clocks of the wasps to be more synchronized and comparisons between them would be less noisy (Rosato and Kyriacou 2006). Locomotor activity was measured as described above. The wasps were entrained to LD 12:12 for at least 3 days (at 18.5 ± 1°C) and then switched to DD. A light pulse was delivered at various time points, beginning with the time of lights-off (Zeitgeber time - ZT12) and then every consecutive hour. The light pulse duration was 1-hour with intensity 150-250 lux, the same intensity as used for entrainment. Phase shift was calculated over the first four full days in DD by the BeFly! software, using a cross correlation method. Median of un-shifted rhythmic individuals was used as a reference phase to the ones experiencing the light pulse to calculate the phase shift difference. Only data with maximum peak correlation 0.2 were used for analysis. The Monte Carlo method was used to randomly shuffle the experimental data (100 permutations) to obtain a confidence level of 99%.

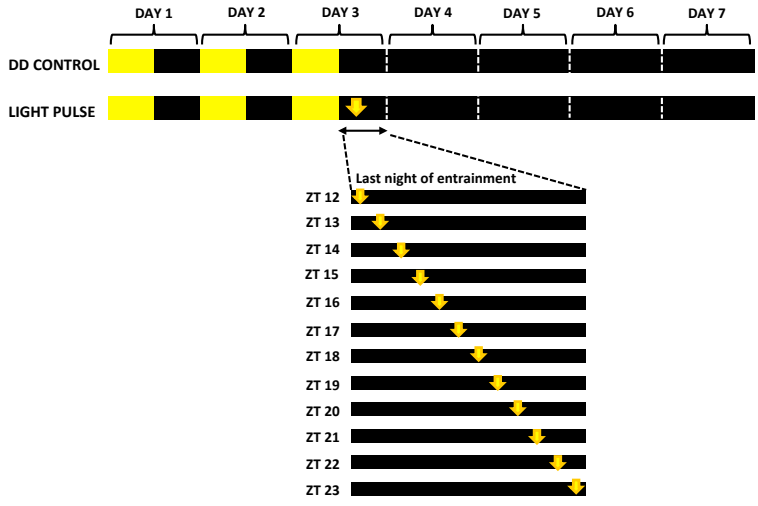


Figure 3.2. Light-dark scheme illustrating anchored protocol for creating phase response curves in *Nasonia*. Each group of 10-90 wasps received a 1-hour long light pulse at different Zeitgeber Time ZT.

Phase shift

The phase response curve revealed the most photo-sensitive time windows of the wasps endogenous clock. I have chosen the time window at the end of the night, where delivering of the light pulse caused a phase advance (Figure 3.4), to test for differences in the response of *Nvcry2* knockdown wasps compared to controls. Wasps were monitored using the DAM system and entrained as described above with anchor protocol and the light pulse delivered at ZT23. The phase shift was calculated using the median of DD (un-pulsed) groups a reference phase.

Re-entrainment (Jet-lag) experiments

The ability to re-entrain to a new LD regime was tested under different light wavelengths by measuring locomotor activity in light boxes with various coloured LEDs. The LD cycle was either advanced or delayed by 6 h with the light of intensity 0.03 $\mu W/cm^2$, on the 4th day after entrainment. The wasps were entrained for three full days in LD 12:12 of white light of intensity 0.25 $\mu W/cm^2$. The phase shift was measured manually as a difference between the time of initiation of activity (referred to as onset of activity) on the last day of entrainment and the second day after phase advance or phase delay (Figure 3.3). Locomotor activity was visualized as double plotted actograms and the phase shift measured with Actogram J plug in for ImageJ (Schmid et al. 2011). The following wavelengths were used: UV (370 nm), blue (464 nm), green (516 nm), yellow (592 nm) and red (using a filter 635 ± 20 nm).

To estimate the effect of individual wavelengths I had to convert the light intensity measured as spectral irradiance (I) [$\mu W/cm^2$] to photon flux [#photons*cm^{-2}*s^{-1}], which is defined as a number of photons per second and unit of area [1/(m^2•s)]. First the photon energies (E_p) for various wavelengths were calculated using equation $E_p = h•(c/\lambda)$; with Plancks constant $h=6{,}63•10^{-34}$ [Js]; Speed of light $c=2.998•10^8$ [m/s]; Wavelength λ [m]. Photon flux for each wavelength was

then calculated as I/E_p. As a following step the mean corrected response was obtained as a mean observed response corrected to the ratio of photon flux for each wavelength relative to photon flux at 370 nm. The relative response was calculated by normalising the individual measured response at each wavelength to the mean corrected response to response at 370 nm.

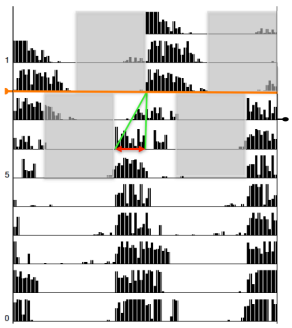


Figure 3.3. Actogram illustrating phase advance (Jet-lag). LD cycle was phase advanced by 6 h (marked by orange line). Phase angle was used to determine the shift in activity-onset between the last day of entrainment and day 2 after LD cycle advance. Red arrow indicates the phase shift (in hours).

RNAi knockdown of Nvcry2

RNA interference (RNAi) was used to investigate the function of *Nvcry2* in circadian rhythms *in vivo.* RNAi for target gene knockdown was performed according to Lynch and Desplan (2006). Double-stranded RNA (dsRNA) was designed to target *Nvcry2* (NV13040) mRNA as indicated in Figure 3.4. The 506 bp target sequence encompasses a larger part of the photolyase-coding region (PLHR). The sequences were verified for presence of any off-target matches using the tool on WaspAtlas (Davies and Tauber 2015).

Nv*cry2* dsRNA was synthetized using the reverse transcribed cDNA of a pooled sample of adult males and a 550 bp dsRNA *gfp* was prepared from plasmid DNA. RNA was extracted with Trizol reagent (Ambion) following the manufacturers protocol. Total RNA was treated with TURBO DNA-free™ DNA Removal Kit (Ambion) to remove contaminating genomic DNA. Quantity and quality of the RNA samples were evaluated with a NanoDrop 2000 spectrophotometer

(ThermoScientific). The first strand cDNA was reverse transcribed from 1 μg of total RNA with SuperScript® II Reverse Transcriptase (Invitrogen), with a 1:6 mixture of Oligo(dT)18 Primer and Random Hexamer Primer (both Thermo Fisher Scientific) using the kit protocol. Remaining RNA was removed from the synthetized cDNA with RNase H (New England Biolabs). cDNA was amplified by PCR on a PTC-100 Peltier Thermal Cycler (MJ Research). The PCR cycle consisted of denaturation at 95 °C for 2 min, followed by 35 cycles of 95 °C for 30 sec, 60 °C for 30 sec, and 72 °C for 45 sec, and a final elongation step at 72 °C for 5 min. PCR was performed with KAPATaq DNA polymerase (KapaBiosystems) in 50 μL volume and target specific primers, *Nvcry2* (forward 5-ACCTTCAGATGCGTCTTCGT-3 and reverse 5-AGCTCCTCG AGGGTAGGAAC-3), *gfp* (forward 5-GGAGACCACCTGAAGTTCATCTGCACCA-3 and reverse 5-GGAGACCACTGCTCAGGTAGTGGTTGTCG-3). PCR products were re-amplified with addition of T7 promoters (TAATACGACTCACTATAGGG), run on a 1 % agarose gel and extracted with MinElute Gel Extraction kit (Qiagen). dsRNA was synthesized with MEGAscript T7 Transcription kit (Ambion) following manufacturers instructions.

For injection of dsRNA, probes were dissolved in nuclease free water (Thermo Fisher Scientific) to a concentration of 1 μg/μL. The injection needle was pulled from a glass capillary with a needle puller - model p80/PC (Sutter Instrument Co.) under the program setting on Heat: 700, Pull: 150, Velocity: 080, Time: 100 and final diameter 7 μm. Eight-day-old *Nasonia* pupae (yellow stage) were used for dsRNA injection. Pupae were collected, attached to a microscope slide with double-sided adhesive tape (Scotch tape) and injected in the side of the abdomen until lightly swollen. A PV830 Pneumatic Pico Pump was used for injections with a pressure of 5-10 mmHg. Pupae were kept in a Petri dish with a piece of cotton soaked in 10% sucrose solution for the wasps to feed on once eclosed.

qPCR for measuring the efficiency of RNAi

The efficiency of RNAi was evaluated by qPCR, using *rpl32* (ribosomal protein) as an internal reference gene. Wasps were kept at 18°C in LD 12:12 and collected five days after emergence and one hour after lights on (ZT1), and snap frozen in liquid nitrogen. Heads were separated and pooled (sample of 20-25 heads) in 4 or 5 biological replicates per treatment. RNA was extracted and cDNA synthesized as described above. Controls with no reverse transcriptase were also tested to check for genomic contamination.

Primers for qPCR were designed with software NCBI Primer-BLAST (Ye et al. 2012) and tested for specificity with gradient PCR. Brilliant® II SYBR® Green Low Rox QPCR Master Mix (Agilent Technologies UK Ltd.) was used to quantify the expression levels of targeted genes in 25 μL reactions with 5 μL of 10x-diluted cDNA. Each sample had three technical replicates. The temperature profile for the qPCR reaction started with activation of DNA polymerase at 95 °C for 15 min, followed by 45 cycles of denaturation at 95 °C for 30 sec and annealing/extension 60 °C for 45 sec. Primers for qPCR were as follows: *rpl32* (product size 145bp): forward 5-GCCCAACATCGGTTATGGTA-3, reverse 5-AACTCCATGGGCAATTTCTG-3; *Nvcry2* (product size 118 bp): forward 5-GGCATGTGGATGTGGCTTTC-3, reverse 5-GCAGGTAACGCCGAATGTAG-3 and *Nvper* (product size 121 bp): forward 5-TCTCGTCGCCTTCTTCCAAC-3, reverse 5-ATCGGGATCGACGTAGGACA-3.

For data analysis, the individual samples were first checked for additional peaks in melting temperature. Threshold cycle values (Ct) of negative control (-RT) as well as the no-control template (H_20) were checked for contamination with genomic or contaminant DNA. Subsequently, Ct-values were compared among the technical replicates and eliminated if differences reached more than ~ 0.5 cycle.

An average of the raw fluorescence of technical replicates for each biological sample was used for statistical analysis with R statistical package qpcR (Ritz and Spiess 2008). Differences in expression levels between treatments were

calculated as a mean ratio of control to treated sample and then evaluated using efficiency and Ct-values of individual samples normalized to the reference gene, *rpl32*. Individual sample Ct-values and efficiency were calculated with sliding window method (Ruijter et al. 2009). Statistical evaluation of qPCR results was done by permutation approach (2000 permutations), applying 95% confidence intervals. This approach shuffles Ct-values and Efficiency values between treatment and control samples, keeping the treatment/control samples tied together. Ratios are calculated and then compared to results obtained from each permutation, where treatment and control samples were reallocated randomly. A p-value is calculated by the number of permutations which obtained a higher/equal/lower ratio than the original data.

Light-dependent luciferase assay

The light-dependent assay was carried out according to Yuan et al. (2007). *Drosophila* S2 cells expression constructs (pAc5.1-*Nvclk*, pAc5.1-*Nvcyc*, pAc5.1-*Nvcyc*ΔC, pAc5.1-*Nvper*-V5-His6 and pAc5.1-His6-*Nvcry2*) with constitutive *actin* promoter from Drosophila gene *actin5C* were created and transfected to S2 cells. Their preparation and transfection methodology is described in Chapter 2. The control transfection contained 50 ng of *Dm*PER 4Ep, 30 ng of pCopia-*Renilla* and empty vector expression vector pAc5.1/V5-HisA. Each sample transfection consisted of 50 ng of *Dm*PER 4Ep, together with 30 ng of pCopia-*Renilla* and 50 ng of each pAc5.1-*Nvclk*, pAc5.1-*Nvcyc*. The luciferase activity was sassed on the presence of pAc5.1-His6-*Nvcry2* (50 ng) or pAc5.1-*Nvper*-V5-His6 (50 ng) or both. Additionally, I measured the effect of E3 ligase JETLAG (JET) on *Nv*CRY2 activity by co-transfection of pAc5.1-His6-*Nvcry2* (50 ng) with 50ng of pAc5.1-*Dmjet.* Construct with *Dmjet* was kindly provided by Lin Zhang (University of Leicester).

The procedure for the luciferase assay was described in Chapter 2. For the light dependent assay cells were transfected in duplicate plates. One replicate was

kept in a light box under constant light conditions (150 lux) at 25°C in a temperature controlled room. The other replicate served as control, plates were kept in the dark by wrapping them in aluminum foil and keeping them under the same conditions as the light exposed ones. The duration of the light exposure was 48 hours, after which the cells were harvested.

RESULTS

Free running period

N. vitripennis circadian rhythm was measured as free running period (τ) of locomotor activity under different light conditions in wildtype individuals. Females had significantly longer τ than males (Supplementary table S2.1) under both light conditions, LL and DD (ANOVA: $F(3, 295) = 78.84$; Tukeys *post hoc* test: $p < 0.0001$). The proportion of rhythmic females was smaller than males in both light conditions (Fishers exact test $_{(LL)}$: $p < 0.001$; Fishers exact test $_{(DD)}$: $p = 0.01$) (Supplementary table S2.1). Another property of circadian behaviour is rhythm robustness, which is defined as the strength and regularity of the rhythm (Refinetti et al. 2007). Females had significantly lower robustness of activity than males under both light conditions (ANOVA: $F(3, 295) = 58.7$; Tukeys *post hoc* test: $p < 0.0001$).

Phase response curve

Light stimulation delivered as a short light pulse during the night causes resetting of the circadian clock in *N. vitripennis* males and females which leads to a phase shift in circadian rhythm, as shown by PRC (Figure 3.4). Phase advance occurs after delivering the light pulse closer to the end of the dark phase (dawn), whereas phase delay is induced by delivering the light pulse at the beginning of the dark phase (dusk). Wasps are not phase shifted by light pulses delivered during the middle of the night.

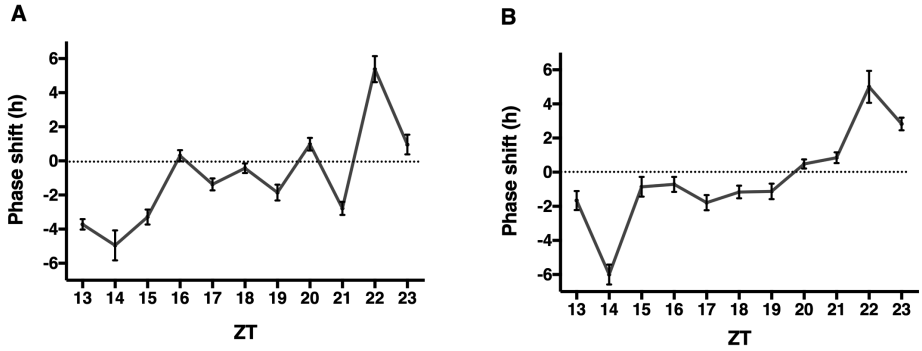


Figure 3.4. Anchored phase-response curve (PRC) of *Nasonia*. Mean ± SEM phase shift for (A) females, n = 10-34 per time point and (B) males, n = 17-90 per time point. Light pulses were delivered separately at ZT13 and every consecutive hour till ZT23.

Effect of Nvcry2 knockdown on the free running period

The structure of the *Nasonia cry2* gene is depicted in Figure 3.5. The gene has 12 exons coding for several main domains such as a DNA photolyase homology region (DNA-PL) and a FAD binding region (FAD-B) (more details about the protein domains were provided in Chapter 2). A 506 bp dsRNA was designed to target the majority of the DNA-PL region (Figure 3.5).

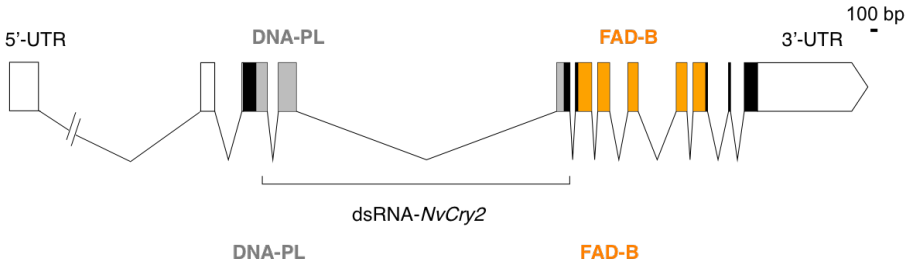


Figure 3.5. Genomic structure of *Nvcry2*. Introns are represented by lines and exons by blocks. White parts correspond to 5UTR or 3UTR regions and coloured parts to coding domain sequence (CDS). Within CDS main domains are highlighted, such as DNA photolyase (DNA-PL) in grey and flavin adenine dinucleotide binding domain (FAD-B) in orange. The region used to design dsRNA *Nvcry2* for RNAi is depicted below the gene. The scale bar on the top right depicts 100 base pairs (bp).

Delivery of *Nvcry2* targeting dsRNA resulted in a significant reduction of expression in heads of males (40%) and females (33%) at ZT1, compared to *gfp* injected controls ($p < 0.05$) (Figure 3.6). The differences in knockdown efficiency could be due to the lower basal level of *Nvcry2* expression in females compared to males (Appendix S2.3). As *Nv*CRY2 was shown to be a negative regulator in the clock feedback loop *in vitro* (Chapter 2), *Nvcry2* knockdown was predicted to effect the expression of other clock genes. *Nvcry2* RNAi affected the mRNA levels of *Nvper* in females ($p < 0.0001$), but not in males (Figure 3.6).

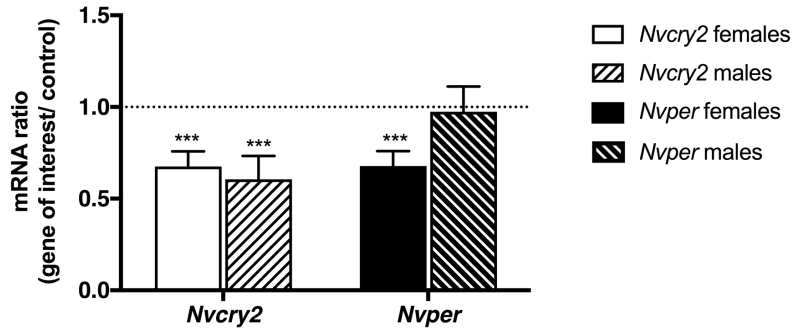


Figure 3.6. Relative expression of *Nvcry2* and *Nvper* in heads of wasps treated with *cry2*-RNAi compared to *gfp*-RNAi controls. Dotted line represents no alteration of mRNA level (ratio 1). Mean ± SEM; statistically evaluated with permutation; *** = $p < 0.0001$; n= 20-25 heads /sample in 4-5 biological replicates.

Overall there was no effect of *Nvcry2* knockdown on the free running period of locomotor activity in females or males, in LL or DD compared to *gfp* RNAi controls ($ANOVA_{(LL)}$: $F(3, 166) = 22.85$; Tukeys *post hoc* test: (females) $p = 0.51$; (males) $p = 0.49$; respectively $ANOVA_{(DD)}$: $F(3, 98) = 2.25$; Tukeys *post hoc* test: (females) $p= 0.89$; (males) $p = 0.99$) (Figure 3.7).

Nvcry2 RNAi affected neither rhythmicity nor rhythm robustness in both sexes under any conditions, with the exception of reduced rhythmicity in the *gfp* RNAi female group in DD (Fishers exact test: $p = 0.02$) (Supplementary table S2.1). *Nvcry2* did not affect the ability of wasps to entrain in LD 12:12 regime (Supplementary figure S2.2).

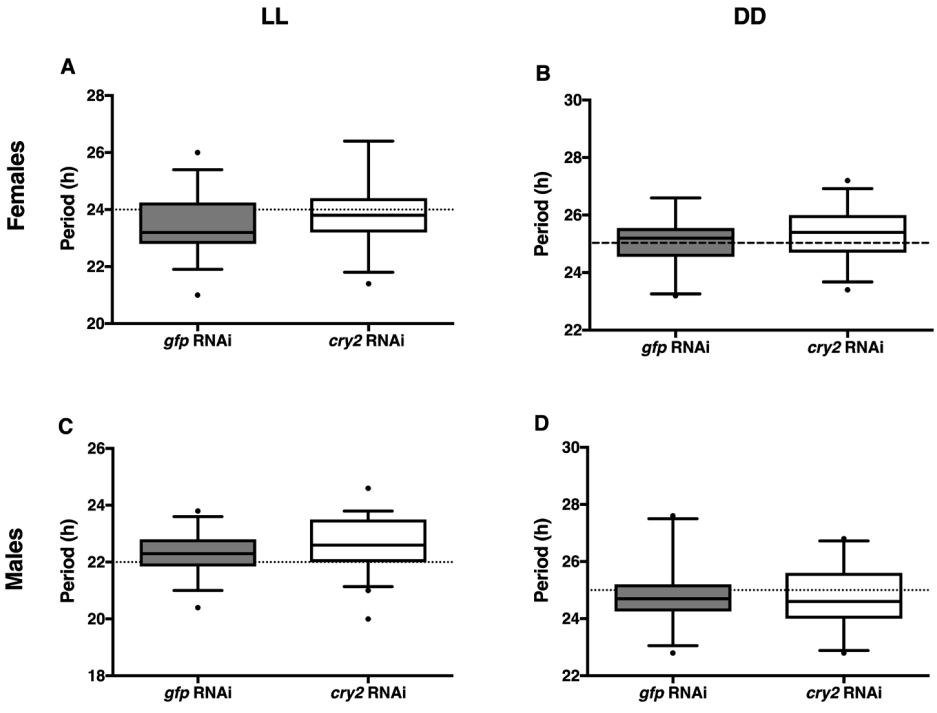


Figure 3.7. Free running period *of N. vitripennis* in *gfp*-RNAi control and *Nvcry2*-RNAi treated wasps. Depicted are box plots with the interquartile range (25–75 percentiles), whiskers corresponding to 5–95 percentiles, horizontal bar to median and dots to outliers. ANOVA; Tukeys *post hoc* test; n = 23-53 per group. Detailed results are provided in Supplementary table 2.1.

Re-entrainment (Jet-lag) experiments

The response of wildtype wasps to phase advance and phase delay of 6 h was tested under different wavelengths of very low intensity (0.03 uW/cm^2). The phase response was measured on day 2 after the light-dark cycle was shifted. I have represented phase shift as a relative response between the measured phase shift induced by each wavelength (A) to expected phase shift (B), normalised to the response induced at 370 nm (Figure 3.8) (for detail explanation see Materials and Methods). The relative effect of each wavelength is determined by how much the relative response differs from 1, the observed/expected ratio (A/B). Phase advance

in both males and females is greater in response to longer wavelengths (> 516 nm with the highest response at 516 nm, which corresponds to the green light ($ANOVA_{(males)}$: F(4, 136) = 37.05; Tukeys *post hoc* test: (464 nm) p = 0.97; (516nm; 592 nm; 635 nm) p < 0.0001; $ANOVA_{(females)}$: F(4, 134) = 31; Tukeys *post hoc* test: (464 nm; 516 nm) p > 0.001; (592 nm) p = 0.01; (635 nm) p = 0.03 (Figure 3.8). Phase delay response at longer wavelengths is not significantly greater than the response at 370 nm. At the longest wavelength tested (640 nm), phase delay response is significantly reduced in both males (ANOVA: F(4, 136) = 4.07; Tukeys *post hoc* test (635 nm): p = 0.0014) and females (ANOVA: F(4, 126) = 5.58; Tukeys *post hoc* test (635 nm): p = 0.001). Thus, phase delay response is more sensitive at lower wavelengths.

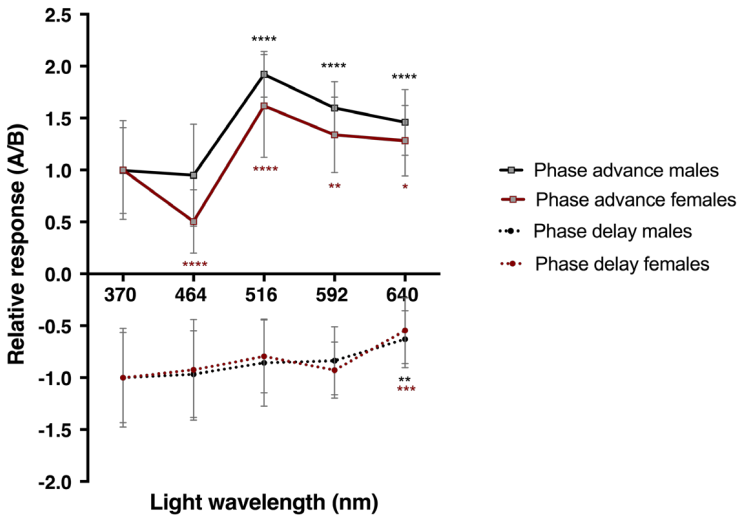


Figure 3.8. Relative response to LD cycle shifted by 6 h in wildtype wasps. Relative response plotted as ratio of the measured phase shift (A) to the expected phase shift (B), normalised to the response at 370 nm for each wavelength after 6 h phase advance or phase delay LD cycle, in males and females on day 2. Light intensity of 0.03 uW/cm^2. Mean ± SD; ANOVA; Tukeys *post hoc* test; **** p < 0.0001, *** p < 0.0002, ** p < 0.002, * p < 0.03; n = 25-40 per light wavelength.

Phase-response of Nvcry2 RNAi wasps

I also tested the phase response of *Nvcry2* knockdown wasps after a light pulse delivered at ZT23. *Nvcry2* RNAi females did not show a significant difference in phase response to the ZT23 light pulse (mean phase shift = 3 ± 1.71 h, compared to *gfp* RNAi controls (2.14 ± 1.93 h) (t-test: t (44) = 1.407; p = 0.1665). In contrast, males showed significantly reduced ability to respond to the light pulse (mean phase shift = 0.6 ± 3.7 h) as compared to *gfp* RNAi controls (mean phase shift = 4.62 ± 3.08 h; t-test: t (76) = 5.183; p < 0.0001) (Figure 3.9).

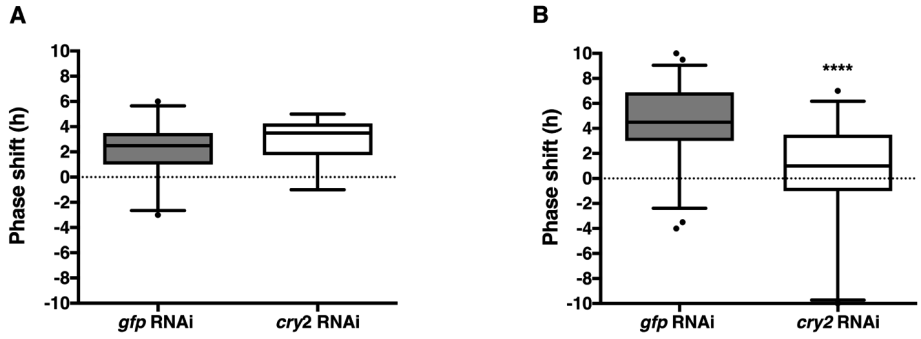


Figure 3.9. Phase shift response after a light pulse at ZT23. *Gfp*-RNAi control and *Nvcry2* RNAi treated wasps. Depicted are box plots with the interquartile range (25–75 percentiles), whiskers corresponding to 5–95 percentiles, horizontal bar to median and dots to outliers for (A) females and (B) males. ANOVA; Tukeys *post hoc* test; **** p < 0.0001; n = 13-48 per group.

The ability of *Nvcry2* knockdown wasps to re-entrain to a new LD cycle under dim light of different wavelengths, was also measured to further investigate the potential role of *Nvcry2* in photoreception, as *Drosophila* type CRY is known as a blue light photoreceptor (Stanewsky et al. 1998). Low intensity (0.25 uW/cm^2) light was delivered to phase advance the LD cycle by 6 h. Knockdown of *Nvcry2* did not significantly affect sensitivity to any of the wavelengths tested (ANOVA: F(9, 153) = 22.11; p_{370nm} = 0.94; p_{464nm} = 1.0; p_{516nm} = 0.48; p_{592nm} = 1.0 and p_{white} = 1.0) (Figure 3.10).

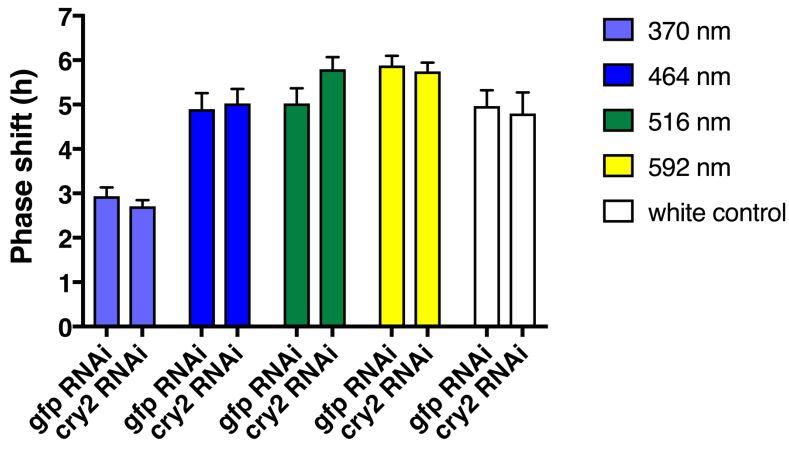


Figure 3.10. Re-entrainment to LD cycle in RNAi-treated wasps. Phase shift response of *gfp* RNA*i*-treated and *Nvcry2* RNAi-treated wasps to advanced LD cycle by 6 h under various wavelengths with intensity of 0.25 uW/cm^2 on day 2. Mean ± SEM; ANOVA with Tukeys *post hoc* test; n= 18-31 per group.

Light dependent luciferase assay

In Chapter 2 I showed that *Nv*CRY2 acts as a transcriptional repressor of *NvCLK:NvCYC* in the core clock negative feedback loop, in a *Drosophila* S2 cell model. To test the hypothesis of *Nv*CRY2 playing a dual role as a photoreceptor as well as a core component of the clock (Sancar 2003; Cashmore 2003), the light dependent Luciferase Reporter Gene Assay was performed (Yuan et al. 2007). If *Nv*CRY2 were to be light sensitive, it would be expected to undergo light-dependent proteasomal degradation (Peschel et al. 2009b), thus reducing repression of *Clk, cyc* and therefore *per* transcription. No difference in the level of transcriptional repression caused by *Nv*CRY2 was observed between constant light (LL) and constant darkness (DD) conditions (Figure 3.11A).

In *Drosophila*, JETLAG (JET) (E3-ligase) causes light-dependant CRY1 degradation *in vivo* and in S2 cells (Peschel et al. 2009b), but it also causes degradation of CRY2 in other species (Zhang et al., unpublished). *Dm*JET did not

affect the inhibitory function of *Nv*CRY2 and therefore did not cause its light-dependent degradation (Figure 3.11B). Overall, these results indicate that the transcriptional repressor function of *Nv*CRY2 is not light-dependant, within the S2 cell *in vitro* model.

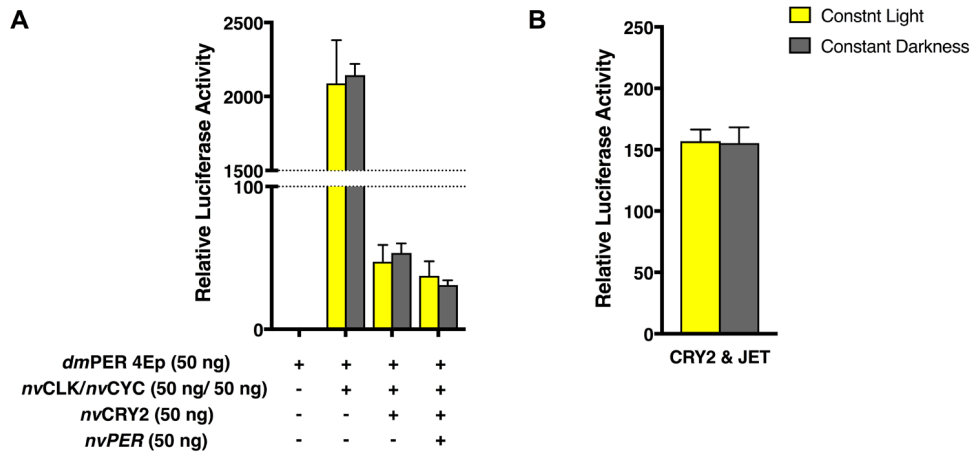


Figure 3.11. Light-dependence as measured in the Luciferase Reporter Assay. (A) *Drosophila per* E-box luciferase reporter (*Dm*PER 4Ep) was used in presence (+) or absence (-) of *Nv*CLK/*Nv*CYC, *Nv*CRY2 and *Nv*PER (mass in ng indicated in brackets). (B) *Drosophila per* E-box luciferase reporter (*Dm*PER 4Ep, 50 ng) was used in presence of *Nv*CLK/*Nv*CYC (50 ng), *Nv*CRY2 (30 ng) with addition of *Dm*JET (50 ng). Luciferase activity was calculated relative to *Renilla* Luciferase. Each value is mean ± SD with independent transfections; ANOVA; Tukeys *post hoc* test; n = 3 per group.

DISCUSSION

Whether or not *Nv*CRY2 is light-sensitive is particularly interesting as hymenopteran insects lack *Drosophila*-type CRY1, which acts as a photoreceptor. Hymenoptera also lack TIM (Rubin et al. 2006; Davies and Tauber 2016), through which light information is relayed to the clock in other species, as well as PTEROPSIN, which is a candidate photoreceptor in the honey bee (Velarde et al. 2005). This study aimed to reveal any role of *Nv*CRY2 in circadian light-

entrainment, based on behavioural assays and *in vivo* gene knockdown experiments. I also examined *Nv*CRY2 light-sensitivity *in vitro* with an S2 cell luciferase assay.

RNAi has already been successfully used in *Nasonia* (Lynch and Desplan 2006; Verhulst et al. 2010). In this study, I performed several behavioural assays after *Nvcry2* RNAi knockdown. Locomotor activity was assayed as it serves as an indicator of circadian rhythms. First, I assessed the effect of *Nvcry2* RNAi treated wasps on entrainment under LD regime to find out if *Nv*CRY2 is involved in light-entrainment. Reduction in *Nvcry2* mRNA levels did not lead to any change in entrainment to LD regime. Similar results were obtained with *D. melanogaster* mutant cry^b (Stanewsky et al. 1998), the cricket *Gryllus bimaculatus* (Tokuoka et al. 2017) and the cockroaches *Blattela germanica* and *Periplaneta amaricana* (Bazalova et al. 2016), after *cry2* or *cry1* RNAi knockdown. This suggests the presence of other light sensitive pathways of circadian entrainment in these species and in *Nasonia*. Supportive evidence for additional light reception pathways was found in *Drosophila*, where employment of visual mutants revealed that not only CRY1, but also the visual pathways play a significant role in light entrainment (Pearn et al. 1996; Stanewsky et al. 1998; Emery et al. 2000; Helfrich-Förster et al. 2001; Schlichting et al. 2015). In fact, the visual system is considered as the major component of the light-mediated entrainment in some species, such as crickets (Komada et al. 2015).

Nvcry2 knockdown males are not able to phase shift after a light pulse delivered at ZT23. This is also similar to the behaviour observed in *Drosophila* cry^b mutants (Stanewsky et al. 1998). However *Nvcry2* knockdown females did show an ability to phase shift. This might be due to the lower knockdown efficiency observed in females compared to males, or due to the lower sample size used in female experiments. On the other hand, the difference in phase shift response between males and females might be because the mRNA level of *Nvper* was not significantly affected after *Nvcry2* RNAi in males, whereas it was in females. This suggests a difference in clock function between males and females. This is

consistent with Bertossa et al. (2013) who showed that the locomotor activity behaviour of the wasps differs between the sexes. There could be various mRNA splicing mechanism of *Nvcry2* between males and females, from which different regulation of circadian clock could originate. Reemploying the luciferase assay in S2 cells but with *Nvcry2* cloned from females instead of males could help to answer some of these questions.

I tested the response of the wasps to a low light intensity of blue light (0.25μW/cm^2) after *Nvcry2* RNAi, based on the fact that *Nv*CRY2 could play a similar role to the *Drosophila*-like CRY, which is considered as a blue light receptor (Stanewsky et al. 1998). *Nvcry2* knockdown wasps were able to entrain to a 6 h advanced light-dark cycle similarly to controls. This is again in agreement with results from the *Drosophila cry^b* mutant, which is able to entrain to shifted LD cycle of dim blue light (Stanewsky et al. 1998). However, in *norpA^{P41}* (mutation causing unresponsiveness of compound and ocelli to light) *cry^b* double mutants, the ability to entrain to shifted LD was considerably lower, or disappeared completely. With no such visual mutants available in *Nasonia*, it is not possible to use the same strategy to test the impact of the visual system on circadian light-entrainment.

It is possible that circadian light sensitivity in *Nasonia* is mediated by photoreceptors of the visual system, as discussed above, or additionally, yet unknown, pathways. The phase advance and phase delay response mechanism has been studied in more detail in *Drosophila* (Peschel and Helfrich-Förster 2011; Yoshii et al. 2016). It was shown that the CRY-dependent pathway acts through sets of neurons called E (evening) neurons, which are the fifth s-LNv and LNd neurons (Grima et al. 2004; Stoleru et al. 2004). However, the E neurons receive light information from the visual pathways as well, and therefore are able to respond to phase shifts even in absence of CRY. Another set of neurons, the M (morning) neurons (l-LNv and LNv neurons) cannot entrain in the absence of CRY and they are not capable of doing so just with the visual light information (Yoshii et al. 2015). The M neurons are responsible for the phase advance response, while E neurons are responsible for the phase delay response (Peschel and Helfrich-

Förster 2011). My results differ here from what was found in *Drosophila*, regarding the necessity of *cry* in phase advance response.

It seems that *Nasonia* phase advancement relies more on long wavelengths light, particularly around 516 nm, which corresponds to the green light. On the other hand, an opposite response is seen in phase delay, where the highest sensitivity is in the lower wavelengths. However, action spectra experiments would be necessary to confirm these findings. Responsiveness to higher light wavelengths was found by Saunders (1974), who showed that *Nasonia* is able to distinguish between 12 and 18 h of red light (> 600 nm) in order to set a photoperiodic response. Saunders (1975) determined the action spectra for photoperiodic induction by exposing wasps to a 13 h period of white light preceded or followed by a 3 h period of light of various wavelengths to simulate dawn or dusk transitions. He found that wasps were most sensitive to wavelengths between 554-586 nm, but also sensitive to 653 nm. It is therefore possible that the photoreceptors for photo-entrainment are similar for seasonal and circadian rhythms.

After silencing *Nvcry2* the wasps did not differ in the length of the free running period or rhythmicity from *gfp* controls. If *Nv*CRY2 participates in the clock as a negative transcriptional regulator (Yuan et al. 2007) one would expect that silencing the gene would cause a change in the free running period (variation in length) and rhythmicity (leading to arrhytmicity) in constant light conditions. However, these expectations were not supported by my results. This could be due to an insufficient knockdown of the gene. The efficiency of RNAi knockdown was 33% reduction in females and 40% reduction in males for *cry2* mRNA at ZT1. However, the levels of the knockdown would vary depending on the circadian phase as *Nvcry2* shows circadian oscillation in expression levels in females (Bertossa et al. 2014). The lower efficiency of RNAi in females might be caused by overall lower amount of *Nvcry2* transcript as compared to males. An alternative explanation for the lack of *cry* knockdown effect on rhythmicity could be the existence of an additional CRY2-independent feedback loop, which would allow the

clock to run even without sufficient contribution of CRY2. Tokuoka (2017) provides such an explanation for the clock in the cricket G*ryllus.* Future experiments should aim for complete knockout of the gene to remove the uncertainty caused by low knockdown efficiency. This could be achieved by the CRISPR/Cas9 technique that is already available for *Nasonia* (Li et al. 2016).

*Nv*CRY2 photosensitivity was also investigated by the light dependent *in vitro* assay in S2 cells (Yuan et al. 2007). Light exposure did not affect the ability of *Nv*CRY2 to inhibit the expression of the reporter either alone or in combination with *Nv*PER. This is similar to results in *Danaus*, *Apis* and *Tribolium* where CRY2 inhibitory function was not affected by light exposure (Yuan et al. 2007). *Drosophila* CRY in S2 cells undergoes light dependent proteasomal degradation mediated through binding with the E3 ubiquitin ligase JET (Koh et al. 2006; Peschel et al. 2009). CRY2 degradation was observed in *Eurydice* after addition of *Dm*JET (Zhang et al., unpublished). However, I have not observed any effect after addition of *Dm*JET in my experiment. Other ligases that are missing in the S2 cells system such as FBXL3 might be necessary for proteasomal degradation of "mammalian-like" CRY.

To conclude, the suggestion of multiple light input pathways into the circadian clock of *Nasonia* is consistent with results from other organisms. This may reflect various evolutionary strategies from adjustment to rapidly changing light intensities and wavelength ranges throughout the day (Stanewsky et al. 1998). *Nv*CRY2 involvement in circadian response can be inferred from the light pulse experiments in males. However, the function of *Nv*CRY2 as a photoreceptor in *Nasonia* has not been proven by the results of this study and requires further investigation. The circadian light sensing appears to be mediated via yet unknown photoreceptors. Circadian light input must be mediated via yet unknown photoreceptors, potentially opsins as described in *Gryllus* (Komada et al. 2015).

ACKNOWLEDGEMENTS

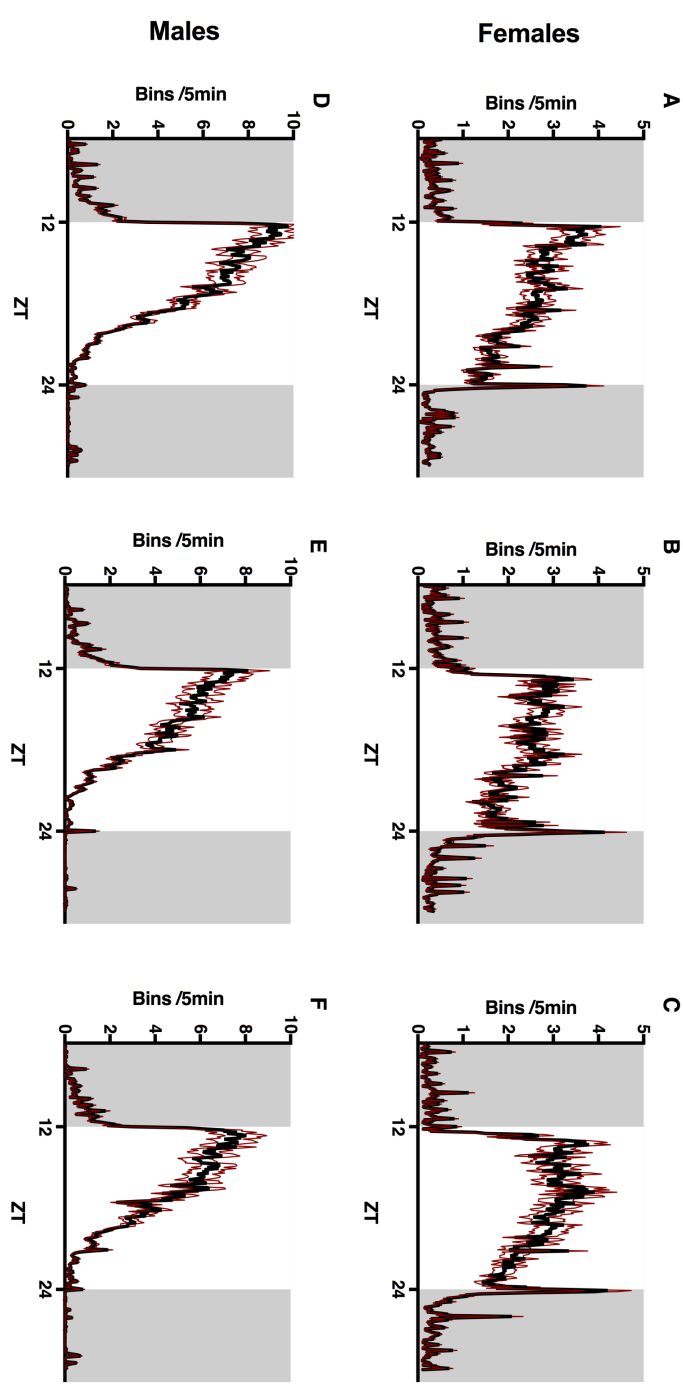
Firstly, I would like to thank to Mirko Pegoraro for introducing me into molecular techniques and data analysis. I would like to thank Nathaniel Davies to show me how to inject wasps and Laura Flavell for sharing her knowledge about RNAi and diapause in *Nasonia* with me. Another thanks belong to summer students who helped me with experiments carried on locomotor activity under various wavelengths, and special thanks belong to master student Anju Joby. Eran for all his suggestions, Roelof Hut for his help with analysis of phase advance and delay. Lastly, thank you all members of INsecTIME for your suggestions and comments.

APPENDICES 2

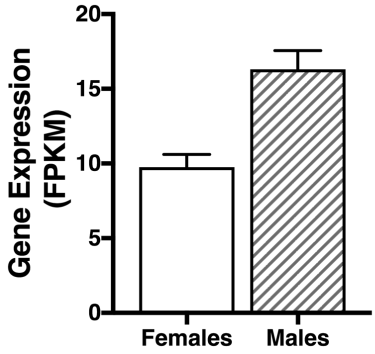
Supplementary table S2.1. Mean ± SD of free running period (FRP), percentage of rhythmic wasps, robustness of the rhythm [(1)] and total number of individuals tested for different treatments, light conditions and sex.

Light conditions	Treatment	Gender	FRP (h)	Proportion of rhytmic wasps (%)	Robustness of the rhythm (%)	Total number of individuals
Constant Light	*cry2* RNAi	Females	23.83 ± 1.22	71.63	19.04 ± 11.95	42
	gfp RNAi		23.52 ± 1.03	67.3	20.25 ± 14.82	52
	intact		23.64 ± 0.18	61	17.79 ± 9.89	59
	cry2 RNAi	Males	22.61 ± 0.89	100	34.26 ± 12.32	53
	gfp RNAi		22.33 ± 0.72	100	35.65 ± 9.54	44
	intact		22.52 ± 0.08	100	39.86 ± 12.27	60
Constant Darkness	*cry2* RNAi	Females	25.33 ± 0.87	97.06	29.4 ± 14.33	34
	gfp RNAi		25.15 ± 0.91	79.17	26.29 ± 12.03	48
	intact		25.02 ± 0.11	93.4	25.13 ± 11.95	61
	cry2 RNAi	Males	24.76 ± 1.1	100	30.38 ± 10.96	23
	gfp RNAi		24.80± 1.01	100	37.82 ± 10.96	25
	intact		24.06 ± 0.09	100	36.62 ± 15.38	119
Constant Dim Blue Light	*cry2* RNAi	Females	23.18 ± 1.08	80.65	17.54 ± 9.68	31
	gfp RNAi		23.08 ± 0.8	82.14	17.11 ± 9.73	28
	cry2 RNAi	Males	23.76 ± 0.62	100	36.53 ± 12.25	29
	gfp RNAi		24.02 ± 0.77	100	38.82 ± 8.960	18

(1) Individual percentages of robustness of the rhythm calculated as a mean with ±SD.



Supplementary figure S2.2. Daily activity profiles at LD 12:12 represented with mean (black line) with ± SEM (thinner red line) of *Nasonia* in bins/ 5 min on a time scale of 24 h. Grey zone represents dark phase and white zone light phase.



Supplementary figure S2.3. Expression level of *Nvcry2* in heads of females and males. Expression data were obtained from RNAseq studies in *Nasonia* WaspAtlas database, expressed as Fragments Per Kilobase Million (FPKM) ± SD (Davies and Tauber 2015). Expression data of *cry2* mRNA most abundant variant XM_001606355.3.

CHAPTER 4

Genome-wide association study of diapause propensity and circadian rhythmicity in Nasonia vitripennis

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ABSTRACT

To investigate the genes involved in molecular regulation of diapause and the circadian clock in the wasp *Nasonia vitripennis*, I carried out a genome-wide association study (GWAS). I used 34 isogenic lines from the *Nasonia vitripennis* Genetic Reference Panel (NVGRP) established from the genetically highly variable HVRx population collected in the Netherlands. Phenotypic screens of these lines revealed substantial phenotypic variance for both the proportion of diapausing broods of females (diapause propensity) under short photoperiod (8 h light) and circadian free running period under either constant light or constant darkness. GWAS revealed several loci that were significantly associated with diapause propensity, but none associated with free running period. This implies that the variation in diapause propensity within the HVRx population is partly explained by genetic variation, whilst variance in free running circadian activity is caused by stochastic and environmental factors.

INTRODUCTION

Seasonal fluctuations in light-dark cycles, temperature, food availability, and the interaction between these variables has led to species-specific adaptations (Hodek 1983; Tauber et al. 1986). Insects are the most diverse group of organisms on Earth and consistent with the diversity of environments which they inhabit, show considerable variation in their response to seasonal changes (Meuti and Denlinger 2013). Insects avoid harsh seasonal conditions by migrating, or entering a physiological state of dormancy called diapause. Diapause is a species-specific response, which leads to arrest in development and growth, or in reproduction (Koštál 2006).

The most reliable environmental cue for the upcoming season is the change in day length, referred to as photoperiod (Denlinger 2002). Many organisms possess mechanisms that measure changes in photoperiod with a so called "seasonal timer" (Koštál 2011). Photoperiodic response consists of multiple steps, including a timing mechanism of photoreception, i.e. measuring the night length (or day length in some species), and a mechanism that counts the number of inductive photoperiods and the downstream regulation of, for instance, hormone levels that effectuate the diapause induction (Saunders 1981; 2014). Despite extensive research on the physiology of insect photoperiodic diapause, little is known about the underlying genes and molecular mechanisms involved in the seasonal timer (Koštál 2011). In particular, the possible role of the circadian clock in photoperiodism has been highly debated. In the past, the involvement of the circadian clock (or clock genes) in photoperiodic regulation of diapause in insects was explored via different experiments, such as classical resonance (Nanda-Hamner), and light interruption during night phase (Bünsow) (Meuti and Denlinger 2013). Nanda-Hamner experiments carried out in *Nasonia vitripennis* suggested the involvement of two separate oscillators in diapause programming, operating at

dawn and dusk (Saunders 1974). The two-oscillator model is known as the internal coincidence model proposed by Pittendrigh & Minis (1964).

Geographical clinal variation in several aspects of photoperiodic diapause induction has been reported for *N. vitripennis* (Paolucci et al. 2013). After exposure to a specific number of days (counter), referred to as switch point, of critical day length (timer), females start to produce various proportions of diapausing larvae, referred to as diapause propensity (Paolucci et al. 2013). The timer, counter of photoperiodic cycles and diapause propensity all show clinal-associated variation (Paolucci et al. 2013), with a genetic component (Paolucci et al. 2016). A QTL study revealed two genomic regions associated with diapause induction in *Nasonia*: a region on chromosome 1, that includes the clock genes *period (per)* and *cycle (cyc)* and a large region on chromosome 5 that includes *cryptochrome2* (*cry2)*. Further analysis revealed that different *per* haplotypes were associated with the clinal variation in the switch point of diapause induction, (Paolucci et al. 2016). In addition, a recent study revealed that *per* knockdown in *Nasonia* females led to disruption of diapause (Mukai and Goto 2016). This suggested that the gene *period* is involved in regulation of seasonal timing.

(Dalla Benetta 2017) showed that *Nasonia per, Clk*, *cyc and cry2* are differentially expressed in northern and southern *Nasonia* populations. This variation in gene expression might be linked to variation in diapause propensity, as was shown in studies of other insect species. For example, in the linden bug *Pyrhocorris apterus*, differential expression of the clock genes *Clock* (*Clk*) and *period* (*per*) was correlated with diapause (Syrová et al. 2003). RNA interference demonstrated the involvement of the clock genes *per*, *cryptochrome2* (*cry2*), *Clk*, and *cycle* (*cyc*) in diapause in the bean bug *Riptorus pedestris* (Ikeno et al. 2010; Ikeno et al. 2011a, 2011b; Ikeno et al. 2013). Whether the effect of *per* and other clock genes on the seasonal response is via the clock mechanism or by a clock-independent mechanism (pleiotropy), is still an open question (Emerson et al. 2009).

In this study, the wasp *Nasonia vitripennis*, an emerging model organism in evolutionary biology was used (Werren and Loehlin 2009). *N. vitripennis* is a cosmopolitan species with a maternally induced facultative larval diapause (Saunders 1965). The *N. vitripennis* Genetic Reference Panel (NVGRP) is a collection of 34 iso-female lines that were generated from the HVRx genetically diverse outbred laboratory population (B.A. Pannebakker, personal communication) based upon wild strains collected in the Netherlands (van de Zande et al. 2014). The genomes of all lines were fully sequenced, providing genome-wide polymorphism information. HVRx and NVGRP are freely accessible as a community resource for analysis of population genomics and genome-wide association (GWAS) mapping of quantitative traits (B.A. Pannebakker, personal communication; van de Zande et al. 2014). The NVGRP lines are isogenic and therefore genetic variation within the lines can be assumed to be absent (V_G=0).

GWAS was employed to identify Single Nucleotide Polymorphisms (SNPs) associated with diapause propensity and circadian free-running period, using NVGRP iso-female lines. Diapause propensity was measured as a proportion of diapausing broods under conditions of short day (LD 08:16) whereas circadian rhythmicity was measured as the free running period under constant light or darkness. No non-synonymous clock gene variants were identified in NVGRP, therefore any SNPs identified would implicate genes and regulatory pathways involved in upstream or downstream regulation of the circadian clock.

MATERIALS AND METHODS

Wasps strains and rearing conditions

The *Nasonia vitripennis* Genetic Reference Panel (NVGRP) of strains was used in all experiments. The HVRx population was established as a balanced mixture of five *N. vitripennis* strains isolated from the Hoge Veluwe National Park in the Netherlands, within a 20 km radius of longitude 5.3 E and latitude 52.1 N. It was

kept under a specific breeding regime to maximize maintenance of genetic variation (van de Zande et al. 2014). 34 iso-female lines were established from the HVRx followed by 9 generations of sib-mating, which resulted in the NVGRP (B.A. Pannebakker, personal communication). For my study, I maintained the iso-female lines under a LD 12:12 (light-dark) cycle at 25 °C in glass or plastic vials (70x20 mm). Lines were maintained on a 14-day culture cycle with approximately 10-20 females on 30-50 hosts per vial and generation. Pupae of *Calliphora* sp. were used as hosts.

Behavioural assays and statistical analysis

Diapause propensity

The 34 NVGRP isofemale lines were tested for their diapause propensity under diapause promoting short day conditions (LD 08:16). First, the emerged females were allowed to mate for one day and then placed into experimental conditions (18 ± 1°C with LD 08:16; light intensity between 250-550 lux). Females were kept individually in 60 mm x 10 mm polystyrene tubes closed with a cotton plug and provided with two hosts for oviposition (ten females per line). The hosts were replaced on day 2, 5 and 9, and the females were discarded on day 10. Parasitized hosts were kept at 18°C in constant darkness. Diapausing larvae undergo arrest in development, whereas normal larvae continue to develop and emerge after approximately three weeks if kept at 18°C. Diapause was scored by opening hosts after 21 days to count the number of diapausing larvae and non-diapausing developing pupae. For each line, I calculated the proportion of diapausing brood as the ratio diapausing larvae to total brood size. The resulting proportion of diapausing brood (further referred to as diapause propensity) was arcsine-square-root transformed and statistically analysed as described below.

Circadian rhythms

Circadian rhythms of each HVRx isofemale line were established by measuring locomotor activity in both sexes under different experimental conditions (LL and DD). The Trikinetics Drosophila Activity Monitoring (DAM) system was used to measure the locomotor activity. Emerged wasps (24-48 h of age) were placed individually into glass tubes with sucrose agarose gel on one side and a clean cotton plug on the other side. Tubes were loaded into monitors, which were kept in light boxes equipped with white LEDs at 150-250 lux placed in an incubator at 18.5 ± 1.5 °C. Animals were entrained for four full days in LD 12:12, and then placed into either constant light (LL) or constant darkness (DD) for at least 9 days. For each line, two parameters of circadian behaviour were measured: the free-running period (τ) and rhythmicity defined as strength of the rhythm.

τ was calculated with in-house software (BeFly! By Ed Green), implementing the cosine algorithm from Refinetti et al. (2007). Four days in LL (or DD) were used to calculate the period (omitting the first day of constant conditions as a transition day) as well as the rhythm strength (F), which correspond to the best fit of the cosine wave. Only rhythmic animals were used to calculate τ. Wasps showing bimodal activity (with tau < 15 h) were included in the mean calculations for each group after their τ was multiplied by two. Rhythmic individuals were defined as those whose maximal F-value from cosine wave fit was over the 99% confidence interval. Outliers from each group were excluded from analysis based on the ROUT method, with Q=1% (Motulsky and Brown 2006). To stabilize the variance, free-running periods were log10 transformed and statistically analysed as described below.

Statistical analysis

Diapause propensity and free running period under LL and DD, were evaluated separately in R (R Developmental Core Team 2012). The analysis was performed

by fitting linear fixed effect models with the function *lm* and *lme* within the *nlme* package (Pinheiro et al. 2012) or *lmer* within the package *lme4* package (Bates et al. 2014). The grouping per line was considered a random effect. Comparison between the two models was carried out with the likelihood ratio test. A linear mixed-effected model was also used to statistically evaluate the effect of explanatory variables such as sex, light treatment, *Wolbachia* infection and individual SNPs, on the observed phenotype (see below).

The broad sense heritability (H^2) was estimated based on the phenotypic variance between lines σ_L^2 and the error variance for each line σ_E^2. Variance values were estimated according to formula: $H^2 = \frac{\sigma_L^2}{\sigma_L^2 + \sigma_E^2}$

Pearson correlation was applied to transformed phenotypic data from NVGRP isofemale lines. Data were tested for correlation between free running period in both light conditions, and between the free running period and the diapause propensity. The free-running period was further tested for correlation between sexes under both light conditions.

SNP association with the phenotypic traits

The sequencing of NVGRP isofemale lines yielded 205,691 high-quality SNPs, which were filtered for minor allele frequency (MAF) > 2% (B.A. Pannebakker, unpublished data). After applying the linear mixed model (as described above), the resulting p values of individual SNPs were subjected to Benjamini–Hochberg adjustment to derive false discovery rate (FDR), and only SNPs with an FDR < 0.1 were considered for further analysis. SNPs with an FDR < 0.05 were all considered and described, whereas those with 0.05 < FDR < 0.1 were filtered by their effect based upon a SnpEff annotation (B.A. Pannebakker, unpublished data). SnpEff predicts the effect of the variant: high - causing frame shifts, additions or deletion of stop codons; moderate – codon change, insertion or deletion; low – synonymous changes; modifier – changes outside the coding region

(http://snpeff.sourceforge.net). Only SNPs with high or moderate impact were used for further analysis.

Functional annotation of genes containing SNPs identified in the GWAS for diapause propensity was carried out through Gene ontology (GO) analysis. Coding sequences of the genes were downloaded from NCBI (http://www.ncbi.nlm.nih.gov) in FASTA format and processed in Blast2Go software to obtain GO associated terms. Blast2Go runs BLASTX (http://www.ncbi.nlm.nih.gov/BLAST) to compare a gene sequence translated in all reading frames against a protein sequence database, which allows identification of potential translation products of an unknown nucleotide sequence. The database was non-redundant (nr) with Blast Expectation Value (E-Value) set to 1.0E-3. GO terms were associated with the hits obtained by the BLAST search by mapping of homologue sequences to GO terms and predicted protein domains.

Effects of variants in the clock genes *period*, *Clock*, *cycle* and *cryptochrome2* were searched for by SnpEff annotation (B.A. Pannebakker, unpbulished data) (Cingolani et al. 2012). Variants in the gene *period* were compared to those identified by Paolucci et al. (2016).

RESULTS

Variation in diapause propensity

Diapause propensity, as a phenotypic indicator of seasonal adaptation, was measured as the ratio of diapausing larvae to the total brood size on day 2, 5, 9 and 10 (Figure 4.1; Supplementary table S3.1 and S3.2). Not all females started to lay eggs on day 2 and therefore it is hard to properly estimate the level of diapause propensity on that day. Substantial variation in diapause propensity was found on day 5, ranging from 9% to 100% (likelyhood ratio test: $\chi^2_1 = 66.6$, $p < 0.0001$). Diapause propensity increased on day 9 in most of the lines (likelyhood ratio test: $\chi^2_1 = 11.9$, $p < 0.001$) and on day 10 almost all females produced diapausing

offspring only (likelihood ratio test: $\chi^2_1 = 0.39$, $p = 0.53$). There was no effect of *Wolbachia* infection on diapause propensity measured on day 5 (likelyhood ratio test: $\chi^2_1 = 0.9$, $p = 0.35$). The broad sense heritability (H^2) for diapause propensity measured on day 5 was 0.34 ± 0.09 (SE), which indicates that a substantial proportion of the variation in behaviour is explained by genetic variability.

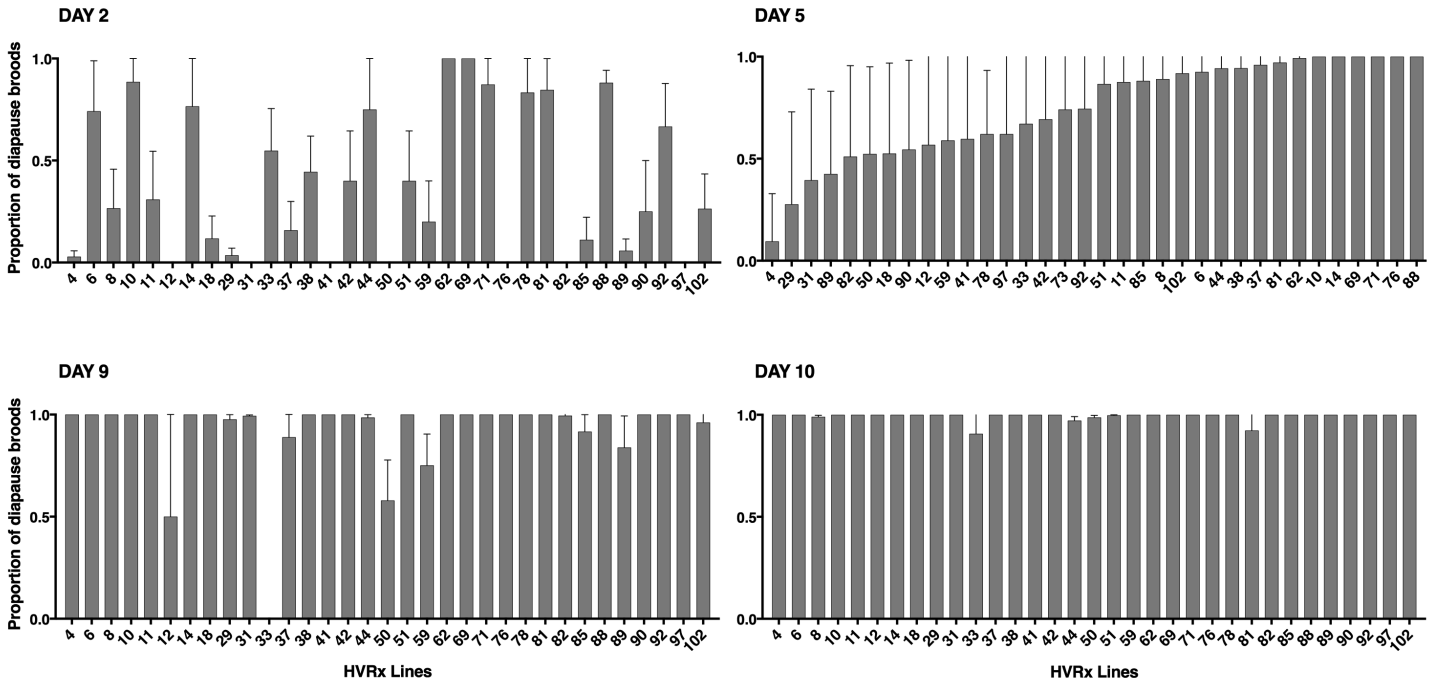


Figure 4.1. Diapause propensity among NVGRP strains of *Nasonia vitripennis*. Females were kept under short day regime (LD 8:16) on day 2, 5, 9 and 10. Mean ± SD; Likelyhood ratio test; n = 2 -17 females per line. Data on day 5 are rank-ordered by ascending mean. Missing data on day 2 for the NVGRP line number 76; on day 9 for NVGRP line number 33.

Variation in free-running period

All isogenic lines had a shorter τ in both males and females in LL compared to DD conditions (Figure 4.2 and Supplementary figure S3.5). The average τ ($\pm$ SD) for females was larger than for males, both under constant light (22.29 $\pm$ 1.02 and 21 $\pm$ 0.70 h) and constant darkness (24.33 $\pm$ 1.69 and 23.35 $\pm$ 1.68 h). There was an appreciable difference in within-line variances. Under constant light, females showed higher levels of within-line variance than males. Sexes showed similar levels of within-line variance under constant darkness, which is overall higher than under constant light. Some lines showed higher levels of within-line variance than others, but there is no obvious tendency for similar lines having higher level of within-line variance in a certain light condition or gender. A significant overall between-lines variation in τ for males and females was observed under both light conditions (likelihood-ratio test; males (LL): $\chi^2_1 = 227.8$; $p < 0.0001$, females (LL): $\chi^2_1 = 271.4$; $p < 0.0001$, males (DD): $\chi^2_1 = 173.514$; $p < 0.0001$, females (DD): $\chi^2_1 = 122.8$; $p < 0.0001$). The effect of *Wolbachia* infection on the free-running period was not significant ($\chi^2_1 = 0.0245$; $p = 0.88$).

The broad sense heritability of the free running period differed across sex and light conditions. The estimated H^2 under LL was 0.28 $\pm$ 4.2e-05 ($\chi^2_1 = 227.8$; $p < 0.0001$) for males and $H^2 = 0.35 \pm 1.0e\text{-}04$ ($\chi^2_1 = 271.4$; $p < 0.0001$) for females. In DD, in males $H^2 = 0.22 \pm 1.2e\text{-}04$ ($\chi^2_1 = 173.514$; $p < 0.0001$) and in females $H^2 = 0.15 \pm 6.6e\text{-}05$ ($\chi^2_1 = 122.8$; $p < 0.0001$).

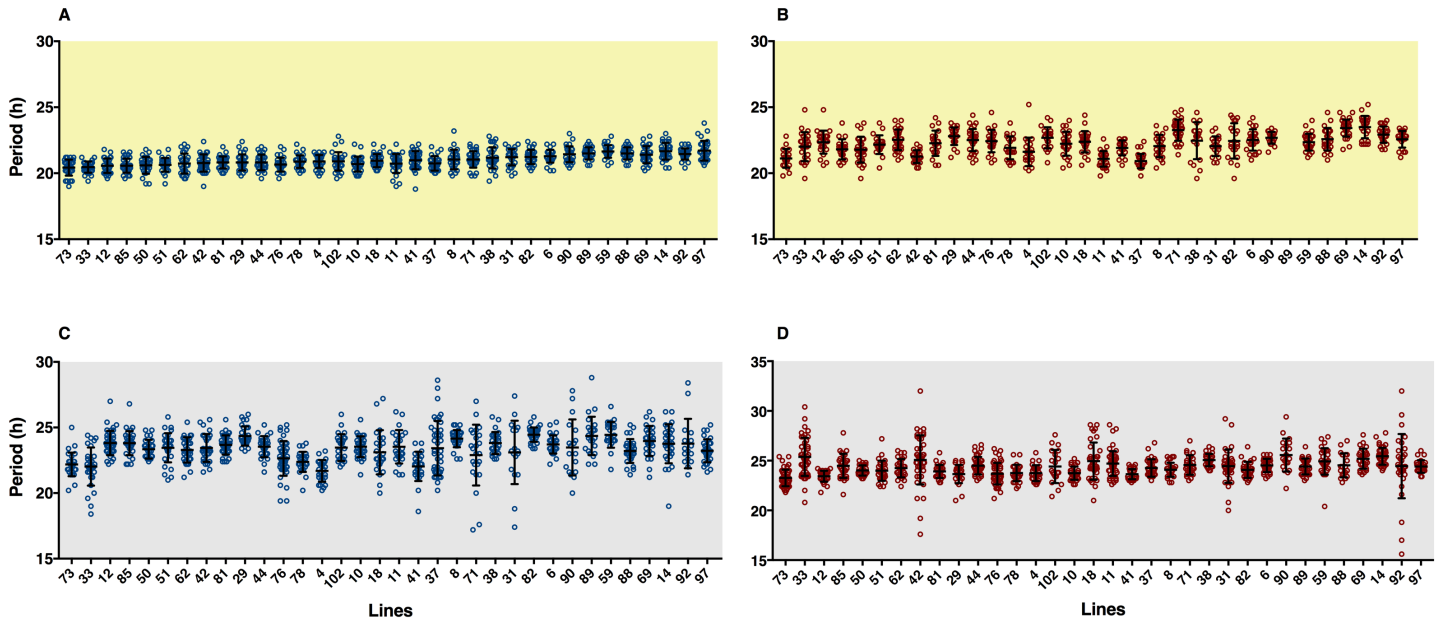


Figure 4.2. The free running period of locomotor activity in NVGRP strains. Wasps were maintained under constant light (LL) or constant darkness (DD) conditions at 18 ± 1°C. Mean ± SD; Likelyhood ratio test; n = 13-45 individuals per line. Outliers were deleted from each group based on the method ROUT (Q=1%). Data are ordered ascending by mean of males in LL. (A) NVGRP males under LL. (B) NVGRP females under LL, (C) NVGRP males under DD, (D) NVGRP females under DD. Graph B has missing data for line 89 due to arrhytmicity in locomotor behaviour.

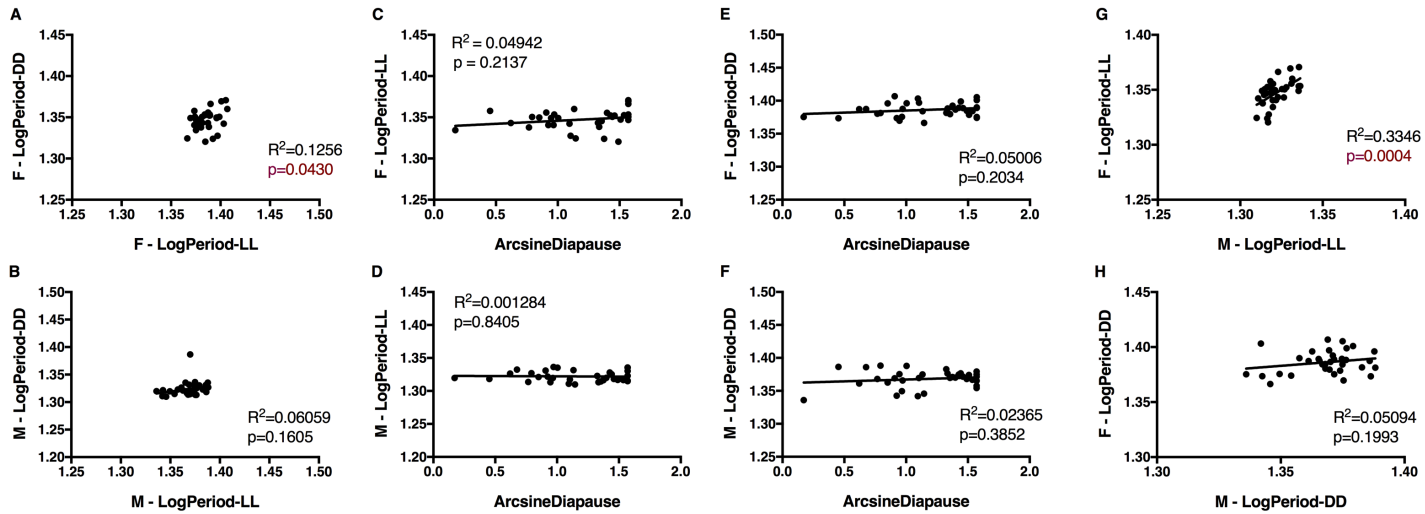


Figure 4.3. Correlation between different phenotypes in circadian and photoperiodic response. The circadian rhythm was measured as the free running period, log10 transformed. Photoperiodic response was measured as proportion of diapausing offspring, arcsine squared root transformed.

Pearson correlation (A) between the free running periods in females under LL and DD, (B) between the free running periods in males under LL and DD, (C) between the free running period in LL and diapause response in females or (D) males, (E) correlation between the free running period under DD and diapause response in females or (F) males, (G) between the free running periods of males and females under LL or (H) DD.

A significant correlation was found between the free running period in LL and DD for females ($p < 0.043$). Positive correlation was also found between the free running period of males and females under LL ($p < 0.04$). No correlation was found between the free running period for males in LL and DD, or the free running period between males and females under DD. No correlation was found between the free running period (under either LL or DD) and diapause propensity (Figure 4.3).

Variation in rhythmicity

Overall HVRx males are more rhythmic than females under both light conditions, but this difference is more apparent under constant light. Under LL, more than 99% of the males are rhythmic, compared to 84% of the females. There is much variation in rhythmicity in females under both conditions and in males under DD, whereas males are mostly rhythmic in LL (Figure 4.4). Overall there is no clear tendency of lines to be more or less arrhythmic across gender or light conditions. Interestingly, females of isofemale line no.89 are completely arrhythmic in LL. On the other hand they do not show high level of arrhytmicity in DD. Wasps from line 89 have somewhat longer free running periods, but lower proportion of diapausing broods on day 5 (Figure 4.1 and 4.2).

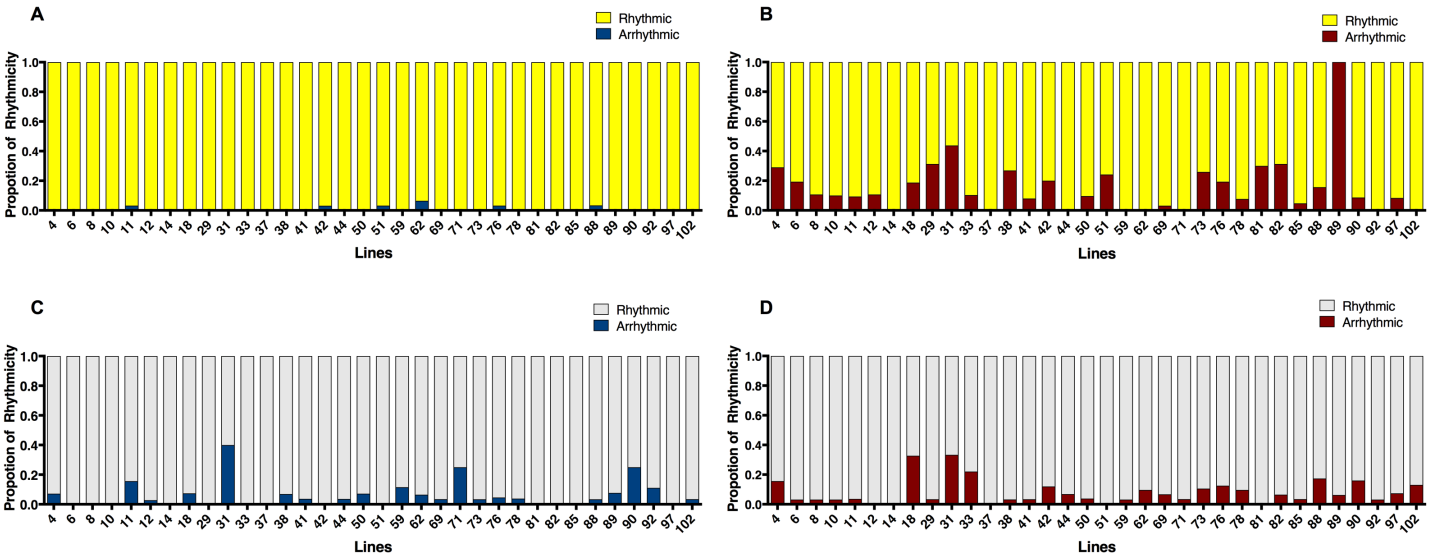


Figure 4.4. Proportion of rhythmic wasps in constant light (LL) or constant darkness (DD). Temperature is 18 ± 1°C and sample size 23-59 individuals. Yellow/Grey part of bar corresponds to a proportion of rhythmic individuals in constant light and constant darkness, respectively; Red/Blue part of bar corresponds to a proportion of arrhythmic females and males, respectively. (A) Males under LL; (B) Females under LL; (C) Males under DD; (D) Females under DD

SNP association with diapause

A total of 816 SNPs were found to be associated with the diapause response at FDR 0.1, from which 13 SNPs reached FDR < 0.05 (Figure 4.5 and Supplementary figure S3.3). The most significant SNPs were located in one region of chromosome 4, where 9 SNPs were part of four genes and 4 SNPs within the 3UTR region of those genes (Table 4.6). These four genes are odorant receptor 175 (Or175), protein CBFA2T1 (LOC100680285), uncharacterized LOC 100119995 (LOC100119995) and signal-induced proliferation-associated 1-like protein (LOC100120131).

Most of the SNP with 0.05 < FDR < 0.1 were intron variants or had low effect - synonymous changes. SNPs with moderate effect (32) and high effect (1) (Supplementary table S3.4) were subjected to GO terms annotation analysis. GO terms were clustered regarding their function and localization as depicted in Figure 4.7. They are reported to be involved in biological processes such as metabolism, protein modification, nuclear division and signal transduction. SNPs were associated with GO terms of regulation on all levels from DNA to RNA to proteins.

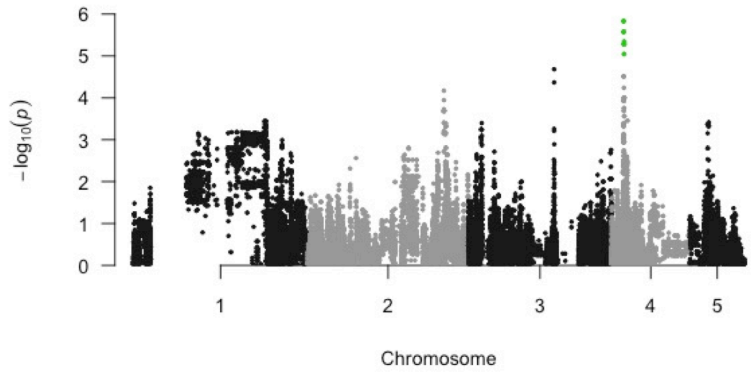


Figure 4.5. Manhattan plot of diapause GWAS. Each point represents a SNP. The position of the SNP along the y-axis represents the strength of association with diapause response, expressed as −log10 (*p* value). The SNPs with FDR < 0.05 are highlighted in green.

Table 4.6. SNPs identified for diapause response filtered by FDR < 0.05. For each SNP the position (BP) on the chromosome (CHR), base alteration (ALT) reference base (REF) and gene identifiers (ID) for Nasonia Base and NCBI are given.

CHR	BP	p-value	REF	ALT	Nasonia Base	NCBI	Gene location	Lenght	Region (5kb)	Gene ontology (GO)	GO term
4	2272005	1.5E-06	G	C	NV23726	100463125	2267225-2270364	3140	-	GO:0050911	detection of chemical stimulus involved in sensory perception of smell
										GO:0004871	signal transducer activity
										GO:0050896	response to stimulus
										GO:0004984	olfactory receptor activity
										GO:0005886	plasma membrane
	2278136	1.5E-06	G	T	-	100680285	2277557-2286481	8925	-	GO:0003700	transcription factor activity, sequence-specific DNA binding
	2280037	1.5E-06	G	T						GO:0045892	negative regulation of transcription, DNA-templated
										GO:0003714	transcription corepressor activity
										GO:0046872	metal ion binding
									-	GO:0006355	regulation of transcription, DNA-templated
	2292153	2.7E-06	C	A	NV11381	100119995	2288655- 2298853	10199	-		
	2300488	2.7E-06	G	A					upstream		
	2301134	2.7E-06	A	T					upstream		
	2301413	2.7E-06	G	C					upstream		
	2350539	4.5E-06	C	G	NV11383	100120131	2321283-2368927	47645	-	GO:0005096	GTPase activator activity
	2351018	5.2E-06	T	G					-	GO:0043547	positive regulation of GTPase activity
	2355140	5.2E-06	C	T					-	GO:0051056	regulation of small GTPase mediated signal transduction
	2356439	5.2E-06	C	T					-		
	2357843	5.2E-06	C	T					-		
	2372916	9E-06	A	G					upstream		

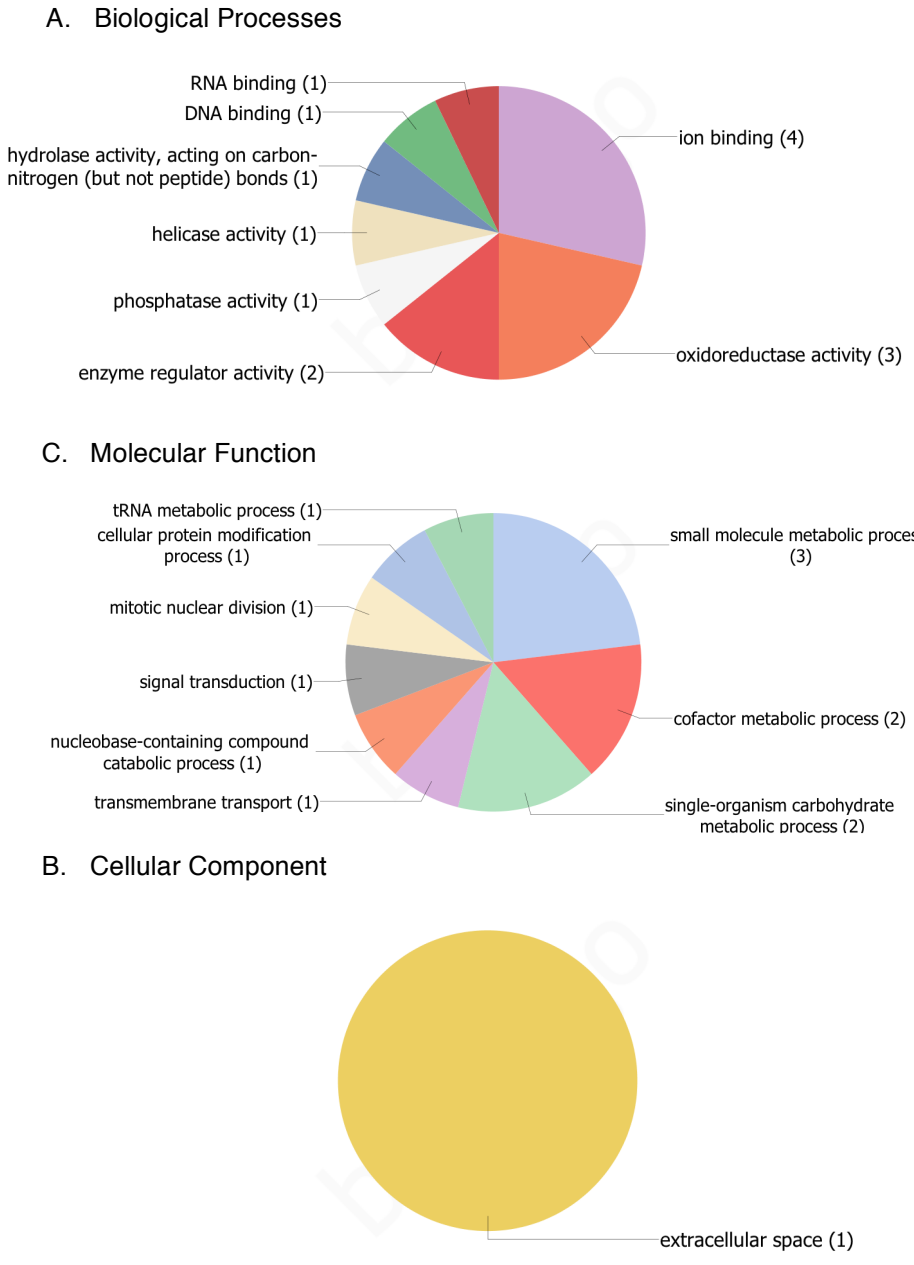


Figure 4.7. Score distribution for GO terms connected with diapause. Genes associated with SNPs with FDR < 0.1 filtered for high and moderate effect in (A) Biological Processes, (B) Molecular Function and (C) Cellular component

SNP association with free running period

Circadian rhythm was measured as the free running period in isofemale lines and used for GWAS. A linear regression mixed model was used to identify the effect of 205,691 variants (B.A. Pannebakker, unpublished data). No SNPs associated with the free running period were found in males or females (under LL or DD) at FDR < 0.1 (Figure 4.8 and Supplementary figure S3.6). No associated SNPs were found under the less strict FDR < 0.2.

No non-synonymous changes were found in clock genes *period, Clock, cycle* and *cryptochrome2* Comparison of the *per* haplotype to previously described *per* haplotypes associated with clinal variation (Paolucci et al. 2016), indicated that only the southern haplotype is present in the NVGRP lines.

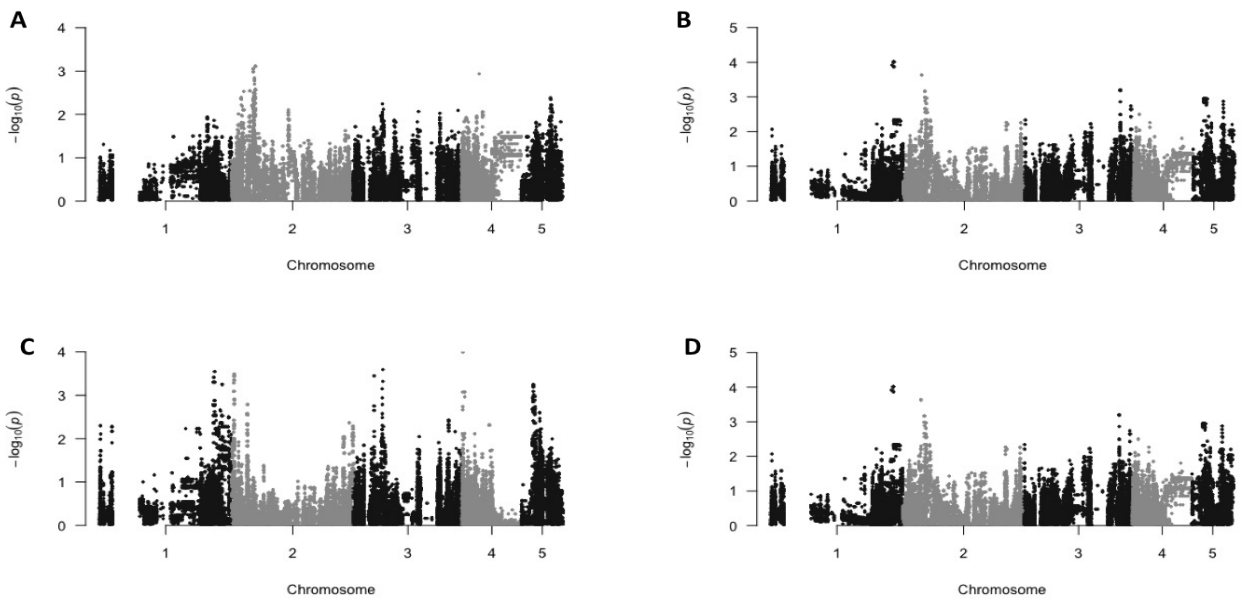


Figure 4.8. Manhattan plots of GWAS for circadian period. The free running period was measured in LL or DD in males and females. Each point represents a SNP. The position of the SNP along the y-axis represents the strength of association with response, expressed as –log10 (*p* value). (A) males in LL (B) females in LL (C) males in DD (D) females in DD

DISCUSSION

In this study, I used a panel of isogenic lines from a genetically variable population of *Nasonia vitripennis,* in order to describe the contribution of genetic factors to variation in diapause propensity and circadian free running period, using a GWAS approach. Diapause propensity was shown previously to be correlated with seasonal adaptation in *Drosophila* (Schmidt et al. 2005a) and *Nasonia* (Paolucci et al. 2013). A comparison between SNPs associated with diapause propensity and circadian rhythms could reveal an overlap in their genetic regulation. Both traits show substantial variation in the HVRx population, however, there was no significant correlation between circadian free running period and diapause.

Variation in phenotypic traits of NVGRP is a result of underlying genetic diversity, phenotypic plasticity (environment) and interaction between genotype and environment (Price et al. 2003). Genetic variation amongst the population is due to occurrence of random mutations and gene flow caused by migration of wasps from other populations. Previous studies showing involvement of the circadian clock / circadian clock genes in the photoperiodic response of *N. vitripennis* were based on datasets containing non-synonymous variants in the clock gene *period*, but most likely also in other clock genes (Paolucci et al. 2016; Dalla Benneta 2017). *Per* was associated with the switch point of photoperiodic diapause induction, a property of the counter of the seasonal timer (Paolucci et al. 2016). The HVRx population, from which NVGRP lines originate, is located centrally to the previously reported latitudinal cline (Paolucci et al. 2016) and therefore would be expected to harbour both northern and southern *per* alleles. The Hamburg population, which is approximately at a similar latitude, contains two *per* hapolotypes, with around 50% frequency of northern haplotypes (~40% of N_1 and ~8% of N_2) and a similar frequency of southern haplotype S_1 (Paolucci et al. 2016). Only the southern haplotype was identified in the HVRx population. The presence of only a single *per* haplotype and lack of non-synonymous variants in

other clock genes could explain the lack of correlation between diapause propensity and the free-running period in this population.

The broad sense heritability of diapause in HVRx females was 0.34. For free-running period, the broad sense heritability varied between 0.15 and 0.35 depending on the gender and light conditions. This indicates that there is a sizable amount of genetic variation for these life-history traits present within the HVRx derived natural populations, which can serve as a precursor for adaptations to changing environment. Estimates of photoperiodic response heritability in other studies were similar. In the pitcher-plant mosquito *Wyeonyia smithii* values between 0.436 to 0.698 were reported (Bradshaw and Holzapfel 1990). Other examples are the ground cricket, *Dianemobius fascipes*in and the moth, *Choristoneura rosaceana,* with a heritability of 0.36 and 0.38 respectively (Shimizu and Masaki 1993; Carrière 1994). A recent study on the leaf beetle, *Orphraella communa,* analysed heritability using a bidirectional artificial selection for diapausing and non-diapausing populations and concluded that this trait is not following simple Mendelian inheritance, but is a polygenic trait. Heritability ranged from 0.77 – 0.86 for the diapausing population and 0.21 – 0.62 in non-diapausing lines.

I observed phenotypic variation not only between the lines, but also within the lines regarding both measured traits. Given that the NVGRP lines are isogenic (no expected genetic variation), any phenotypic variance within the line should be due to the effect of environmental interaction, such as sensitivity to the environment (Lallias et al. 2017), plasticity (Price et al. 2003), canalisation, homeostasis (MØller and Swaddle 1997) and possibly inbreeding depression (Charlesworth and Willis 2009).

Overall 816 SNPs associated with diapause propensity were identified with FDR < 0.1, from which 13 SNPs reached FDR < 0.05. All 13 highly significant SNPs correspond to a small region on chromosome 4, but none of them were non-synonymous variants. Four genes were identified as being associated with those SNPs. The majority of SNPs were predicted to have no modifying effect on gene

function (changes occurring outside of coding regions). The genes identified for SNPs correlated with diapause propensity could be acting upstream or downstream of the seasonal timer, or may have been selected upon their functions in other traits (due to pleiotropy) (Emerson et al. 2009). This might reflect the situation in *Drosophila* where developmental time, stress resistance, lifespan and fecundity were found not only to oscillate seasonally (Behrman et al. 2015), but also to be genetically correlated to diapause propensity (Schmidt et al. 2005b).

I have not identified any SNPs correlated with phenotypes of circadian rhythms. One explanation could be that the variation in the phenotype is not sufficiently robust (insufficient differences between lines) to identify associated SNPs. Additionally, there could be involvement of many genes with small individual effects. Similarly, as mentioned above in case of seasonal response, the variation could result from adaptation of traits correlated to circadian rhythm (Harano and Miyatake 2010). Therefore, the phenotypic variation in the free running period can be explained as a result of environmental or stochastic factors rather than direct selection pressure on the circadian clock.

Among the diapause most significant SNPs is the gene *Or175*, which is involved in the olfactory system as an odorant receptor. Odorant receptors are G-proteins coupled receptors that initiate the sense of smell via reaction with odorous chemicals and then trigger a response through coupled G-proteins (Iwata et al. 2017). Another gene from the same family, the general odorant binding protein 83-like (*Obp03b*), was previously identified in an RNAseq study in *Nasonia* females, and differentially expressed under exposure to either long or short photoperiods (Flavell 2017). RNAi knockdown of this gene caused a significant decrease in the percentage of diapausing progeny. Another protein from the same family, odorant binding protein 1 (*Obp1*), was also identified in a study comparing early diapausing and non-diapausing larvae in *Nasonia* (Wolschin and Gadau 2009). These findings reveal the importance of the olfactory system in regulation of photoperiodic response in *Nasonia,* through different diapause stages.

Another two genes identified in this study are coding protein CBFA2T1 and signal-induced proliferation-associated 1-like protein 1 (SIPAL1L1). The gene ontology terms of CBFA2T1 include transcriptional regulation, which might represent an essential step in response to immediate changes in the environment. The protein was previously studied in connection with acute myeloid leukaemia in a complex with RUNX-1, and involved in proliferation, up-regulation and senescence inhibition (Martinez et al. 2004). SIPAL1L1 is linked to signalling pathways such as in non-canonical Wnt in vertebrates development (Semenov et al.2007) and Rap signalling in cellular processes (Spilker and Kreutz 2010). The precise role of these genes in diapause regulation needs to be further determined.

Several candidate genes that were identified here are involved in biological processes such as regulation of metabolism of carbohydrates, cofactors or small molecules. Many other candidates are involved in regulatory functions, such as E3 ligases or phosphatases. In a comparative study on diapausing larvae and non-diapausing larvae (Wolschin and Gadau 2009) differential abundance of proteins involved in metabolic processes, in particular sugar metabolism and protein regulation, were identified.

Many SNPs identified in this study are located in intron regions. Intronic polymorphisms can have a functional effect (reviewed in Cooper 2010) such as altering splicing donor and receptor sites or modulating chromosome folding (Shepherd et al. 2015; Visser et al. 2012). Therefore, these SNP can be of functional importance and could be explored in greater detail in further studies.

Seasonal adaptation can be regulated on many levels, starting at the photoreception stage. In *Drosophila melanogaster* the circadian photoreceptor *cry* was studied for adaptive polymorphic variants throughout Europe with the expectation that strong selective pressure would act upon a latitudinal cline as day-length change gradually along the latitude (Pegoraro et al. 2014). However, flies carrying different *cry* variants did not show differences in light sensitivity, or a latitudinal cline in Europe (Pegoraro et al. 2014). In *Drosophila*, light perception is mediated through TIM which acts as a binding partner for CRY. Several variants of

tim (*s-tim*, *ls-tim*) were found to have a role in adaptation to seasonal and circadian photo-responsiveness (Sandrelli et al. 2007; Tauber et al. 2007).

A GWAS study on diapause induction in the crustacean, *Daphnia magna*, revealed a candidate gene coding a photoreceptor GPCR rhodopsin and thus highlighted the importance of light perception in the photoperiodic response (Roulin et al. 2013). Comparative GWAS on diapause propensity between individual *Drosophila* collected in spring and autumn during several years revealed the most outstanding polymorphism was within the gene *Crystallin* (Bergland et al. 2014). This gene encodes a glycoprotein that is down-regulated during diapause on both a transcriptional and proteomic level (Zhao et al. 2016). Interestingly, *Crystallin* was identified to be expressed in the corneal lens, the outer structure of fly ommatidia, responsible for focusing light onto photoreceptors (Komoriand et al.1992; Janssens and Gehring 1999). This leads to the suggestion that adaptation to seasonal changes is not centred just in the clock components, but also in regulatory upstream or downstream pathways. However, the functional involvement of identified SNPs in diapause propensity of *Nasonia* require further study.

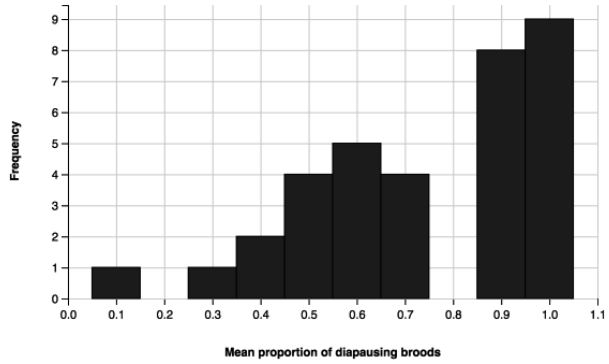
ACKNOWLEDGEMENTS

In this chapter my biggest thanks belongs to Bart Pannebakker for the NVGRP lines, SNPs database and for all the help with analysis and advice. Thank you Gabriella Bukovinszkine Kiss for sending me the waps. Thanks also belong to my supervisors Eran, Louis and Leo for all the advice and help with this chapter. Thank you Rosemary Barnett for all the locomotor activities, diapause experimentns and R analysis. Thank you Charlotte Gale for help with diapause experiments and your enthusiasm. Lastly, thank you all members of INsecTIME for your suggestions and comments.

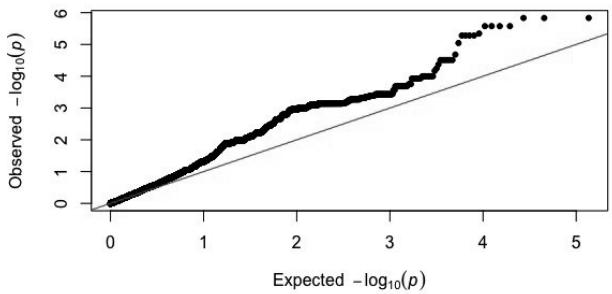
APPENDICES 3

Supplementary table S3.1. Diapause response of HVRx isofemale lines as a proportion of diapause broods in comparison with total number of broods per line on day 5, n = sample size. *Wolbachia* infection is coded as 1 and no infection is coded with 0.

Line	Mean	SEM	n	Wolbachia
4	0.10	0.23	6	1
6	0.93	0.20	17	0
8	0.89	0.16	7	1
10	1.00	0.00	10	1
11	0.88	0.35	8	1
12	0.57	0.47	8	0
14	1.00	0.00	10	1
18	0.53	0.44	13	1
29	0.28	0.45	8	0
31	0.39	0.45	9	1
33	0.67	0.36	8	0
37	0.96	0.11	7	1
38	0.94	0.11	7	1
41	0.60	0.46	10	1
42	0.69	0.40	9	1
44	0.94	0.15	8	1
50	0.52	0.43	9	1
51	0.87	0.26	9	1
59	0.59	0.58	2	1
62	0.99	0.02	6	1
69	1.00	0.00	14	1
71	1.00	0.00	7	1
73	0.74	0.29	10	1
76	1.00	0.00	7	1
78	0.62	0.31	15	0
81	0.97	0.08	8	1
82	0.51	0.45	7	1
85	0.88	0.16	7	0
88	1.00	0.00	14	1
89	0.43	0.41	10	1
90	0.54	0.44	7	1
92	0.74	0.39	7	0
97	0.62	0.42	9	1
102	0.92	0.17	7	1



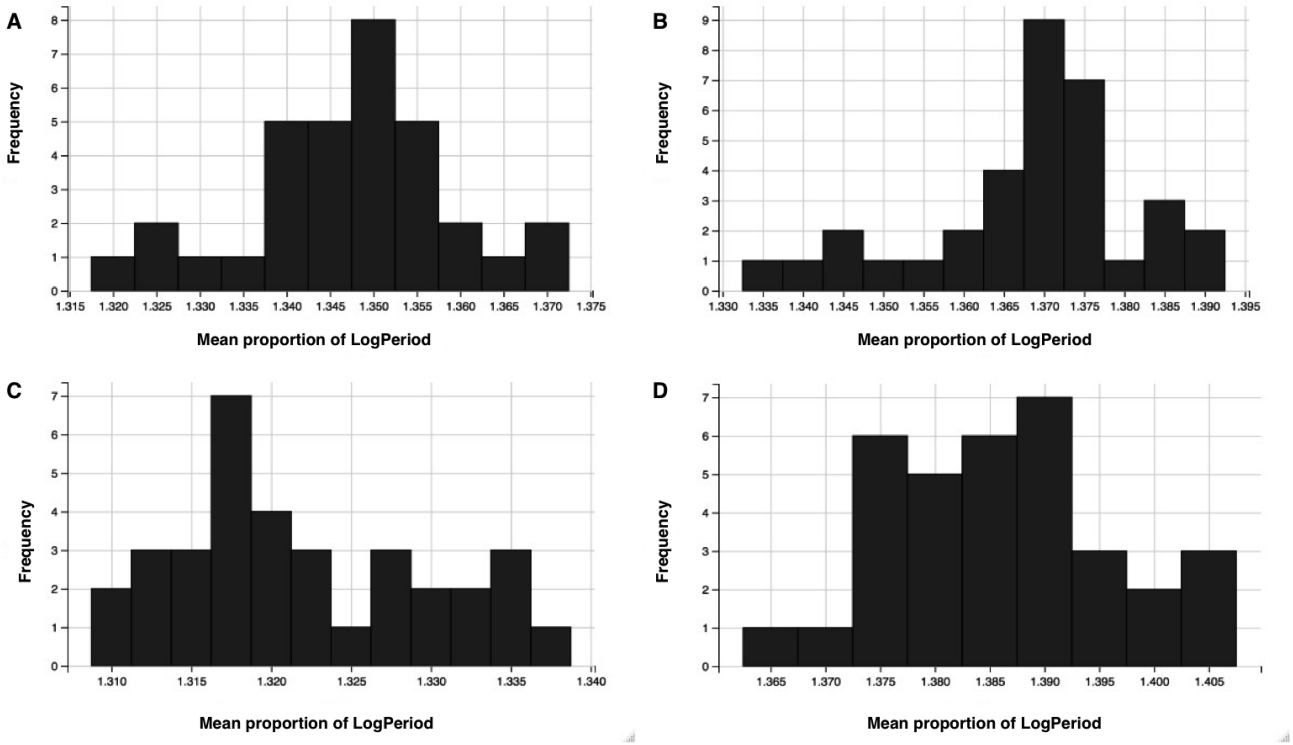
Supplementary figure S3.2. Histogram for the mean of the arcsine transformed photoperiodic diapause measured as a proportion of diapausing broods of NVGRP lines.



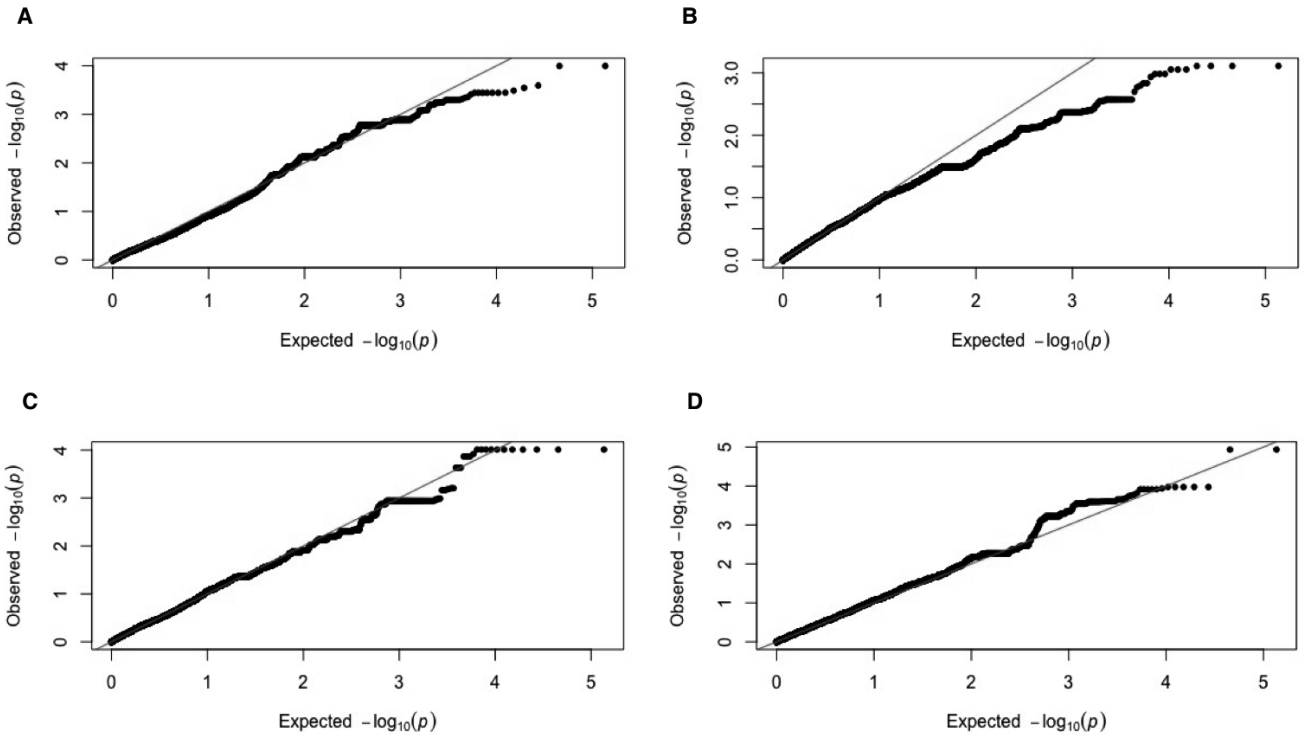
Supplementary figure 3.3. Q-Q plot of the observed vs expected p value for SNPs associated with diapause response.

Supplementary table S3.4. SNPs identified for diapause response filtered by FDR < 0.1 and with effect high or moderate. For each SNP there is position (BP) on the chromosome (CHR) and alteration (ALT) of the SNP, together with the reference (REF) and p-value obtained with linear mixed model association. Effect of the SNP and the allocated gene with identifiers for Nasonia Base and NCBI.

							Gene ID	
CHR	**BP**	**p-value**	**Effect**	**REF**	**ALT**		**Nasonia Base**	**NCBI**
1	**24866000**	5.28E-04	moderate	T	A	missense_variant	NV10027	LOC100118136
1	**24875821**	5.28E-04	moderate	G	A	missense_variant	NV10028	LOC100118176
1	**24988973**	1.08E-03	moderate	C	T	missense_variant	-	LOC103317863
1	**25183900**	1.10E-03	moderate	G	A	missense_variant	NV10047	LOC100119199
1	**25189969**	1.10E-03	moderate	T	A	missense_variant	NV10048	LOC100119234
1	**25360079**	1.15E-03	moderate	C	T	missense_variant	NV10070	LOC100120046
2	**25689541**	1.14E-04	moderate	T	A	missense_variant	-	LOC100680139
2	**25697677**	6.60E-04	moderate	C	T	missense_variant missense_variant	NV14860 -	LOC100119058 LOC103315835
3	**16179919**	5.54E-04	moderate	T	A	missense_variant	-	LOC100679686
4	**2254002**	9.78E-04	moderate	G	T	missense_variant	NV11376	LOC100119762
4	**2269222**	8.46E-04	moderate	C	T	missense_variant	NV23726	LOC100463125
4	**2282384**	4.76E-04	moderate	C	G	missense_variant	-	LOC100680285
4	**2284392**	9.20E-04	moderate	C	A	missense_variant	NV11380	LOC100119965
4	**2287881**	3.09E-05	moderate	G	T	missense_variant	NV11380	LOC100119965
4	**2373553**	1.00E-04	moderate	C	T	missense_variant	NV11385	LOC100120187



Supplementary figure S3.5. Histogram for the mean of the log transformed free running period of males (A, C) and females (B, D) under LL or DD respectively



Supplementary figure S3.6. Q-Q plot of the observed vs expected p value for SNPs associated with the free running period in males (A, C) and females (B, D) in LL or DD, respectively.

CHAPTER 5

General Discussion

This thesis focuses on a non-model insect species, the parasitic wasp *Nasonia vitripennis*, to study both circadian and photoperiodic clock mechanisms. The research aimed at unravelling the molecular basis of the clock that governs rhythmic behaviours and how light entrainment is effectuated. A second main objective was to study the genetics underlying natural variation in photoperiodic response and circadian rhythms, with the goal to identify novel genes related to those phenotypes. I used diapause as a robust light-driven photoperiodic response and the circadian free-running period as a clock phenotype.

Orthologues of the *Drosophila* clock genes *cry1* and *tim1* are missing in *Nasonia,* similar to another hymenopteran, *Apis mellifera* (Rubin et al. 2006). The *Nasonia* clocks bears similarities to the mammalian clock, not only because of the clock gene composition, but also in the behaviour of the genes *NvClk and Nvcyc.* As *cry1* and *tim1* are not present in *Nasonia* (Zhang et al. 2011) and neither is *pteropsin*, which was suggested as the candidate for light entrainment in *A. mellifera* (Velarde et al. 2005), an alternative light entrainment mechanism may exist via *Nvcry2* (Rubin et al. 2006; Yuan et al. 2007). To test this hypothesis, I carried out *in vitro* and *in vivo* experiments to test the role of *Nvcry2* in regulation of circadian rhythmicity in *Nasonia.*

The autoregulatory feedback mechanism of *Nasonia* (Chapter 2) seems to be similar to that in *Apis* (Yuan et al. 2007), suggesting a conserved role of clock genes in the Hymenoptera. *Nv*CRY2 is the main negative regulator of the clock feedback loop mechanism in *Nasonia*, acting independently from *Nv*PER. *Nv*PER on the other hand does not have the ability to inhibit transcriptional activity of *Nv*CLK:CYC alone. *Nv*PER could potentially regulate *Nv*CRY2 stability outside the nucleus, as has been described for the butterfly, *Danaus plexippus* (Zhu et al. 2008). However, whether *Nv*PER creates a complex with *Nv*CRY2 and enters the nucleus still needs to be determined. Interaction assays such as bimolecular fluorescence complementation, co-immunoprecipitation, or yeast-2-hybrid could answer the question as to whether *Nv*PER and *Nv*CRY2 physically interact. The

latter two of these assays could be facilitated using the tagged constructs of *Nv*PER and *Nv*CRY2, which I produced during this study, as there are no functional antibodies currently available for *Nasonia* clock proteins.

Given the role of *Nvcry2* as a transcriptional repressor, one would expect that a knockdown of *Nvcry2* would result in a profound change in the length of τ or level of rhythmicity. However, this effect was not observed, which would suggest that *Nvcry2* is not a repressor. One explanation could be existence of an additional feedback loop independent of *cry2*, which has been demonstrated in crickets. The study of Tokuoka et al. (2017) in the cricket *Gryllus bimaculatus,* suggests that *cry* variants create a feedback loop and repress transcriptional activity of CLK:CYC independently and additionally to repressive activity of *per and tim.* Another explanation is that the knockdown was not sufficient enough to disturb the phenotype.

To investigate the potential role of *Nv*CRY2 as a photoreceptor in *Nasonia in vivo,* I performed behavioural experiments on *Nvcry2* RNAi treated wasps. After *Nvcry2* RNAi males were not able to adjust their behaviour by phase shift to the light pulse delivered at the end of the night as compared to *gfp*-injected control males. However, they were able to entrain to a light-dark cycle and re-entrain to a light-dark cycle of dim blue light that was advanced by 6 hours (Chapter 3). These behavioural experiments gave similar phenotypes as to that of *Drosophila* mutant cry^{b} (Stanewsky et al. 1998). However, to confirm the role of *Nv*CRY2 in photoreception would require inhibition of the visual system as was performed in *Drosophila* (Stanewsky et al. 1998).

Yuan et al. (2007) tested CRY2 light sensitivity in several insect species, including *A. mellifera, D. plexippus* and *Tribolium castaneum, in vitro* through a light-dependent luciferase assay. I used a similar approach to test CRY2 light sensitivity in *Nasonia* (Chapter 3). I found that *Nv*CRY2 was functionally unaffected by light similar to results of Yuan et al. (2007). A possible reason for CRY2 light insensitivity comes from an *in vitro* study by Kutta et al. (2017) who showed that the FAD binding pocket differs between CRY1 and CRY2, leading to weaker binding of FAD to CRY2 than CRY1. For reasons stated above I do not expect

*Nv*CRY2 to be directly involved as photoreceptor in light entrainment and therefore other light pathways are likely to be involved in entrainment of circadian rhythms of *Nasonia.* It is thus necessary to test other candidate genes such as *opsins,* as identified in *G. bimaculatus* (Komada et al. 2015; Chapter 3). With knockout technologies, such as RNAi and CRISPR/Cas genome editing, that are now established in *Nasonia* (Lynch and Desplan 2006; Li et al. 2016), it would be possible to create visually blind mutants to help fully solve the question about *Nv*CRY2 involvement in light entrainment. The other possible pathways such as those involving *opsins* (particularly *long-wave opsin* – see below) can be tested by applying those techniques in behavioural experiments similar to my study of *Nvcry2* knock down. Which photoreceptors are involved in the light entrainment can be investigated indirectly by testing the effect of various wavelengths in phase shift responses.

In *Nasonia,* an action spectrum approach was previously utilised to define wavelengths involved in photoperiodic diapause induction (Saunders 1975). Results pointed towards longer wavelengths, in the red region (> 600 nm). I used a similar approach to identify the wavelengths involved in entrainment of the circadian rhythms (Chapter 3). Both sexes entrained faster under longer wavelengths, particularly around 516 nm. This response might be due to the UV-opsins or other types of photoreceptors, which have not been identified yet. However, a full action spectrum under various light intensities needs to be performed in order to obtain precise information regarding the wavelengths important for entrainment of circadian rhythms.

Another approach to identify possible candidate genes for light entrainment is a genome wide association study (GWAS). This builds on natural variation to identify genetic polymorphisms co-segregating with various phenotypes. My experiments revealed substantial variation in both diapause propensity and circadian rhythms, despite that the used strains originated from one geographical region in the Netherlands. This indicates the presence of standing genetic variation for genes involved in those phenotypes. This variation is not necessarily due to

direct selection on the clock mechanism, but could also reflect pleiotropic effects of those genes on other traits. Several SNPs that show significant correlation to diapause propensity were mapped to four coding regions (Chapter 4).

Variation in diapause behaviour was already described in natural populations of *N. vitripennis* collected from different latitudes in Europe (Paolucci et al. 2013). It was later shown that regional variation in photoperiodic diapause was correlated with polymorphisms in the clock gene *period* (Paolucci et al. 2016). The HVRx population was established from wasps collected in the Netherlands, which is in the middle of the cline of Paolucci et al (2013; 2016). Surprisingly, I have not found similar polymorphisms in the gene *period* within lines of NVGRP as expected from comparison with lines of similar latitude collected in Hamburg, Germany (Paolucci et al. 2016). The candidate genes identified through SNPs correlated with diapause propensity require further study to reveal their possible functional involvement in diapause response.

Concluding remarks

The molecular circuit of the circadian clock in *Nasonia* seems to share the main characteristics with other insect species that possess a “mammalian-like” clock mechanism. In Figure 5.1 I provide the information that we now have about the composition of the Nasonia clock. *Nasonia* CRY2 negatively regulates transcriptional activity of *Nv*CLK:CYC independent from light stimuli. *Nv*CYC posses transactivation domain BCTR, not *Nv*CLK. However, the role and interaction of *Nv*PER with other clock genes, especially *Nv*CRY2 is to be determined *Nv*CRY2 is unlikely to be involved in light perception and therefore other candidates such as long-wave opsins should be investigated for their role in photo-entrainment of circadian clock. In addition, study on diapause propensity in NVGRP revealed several potential candidate genes involved in this phenotype. More study is required to understand the molecular basis of photoperiodic timing in *Nasonia* and this may provide a better understanding of the clock in insects.

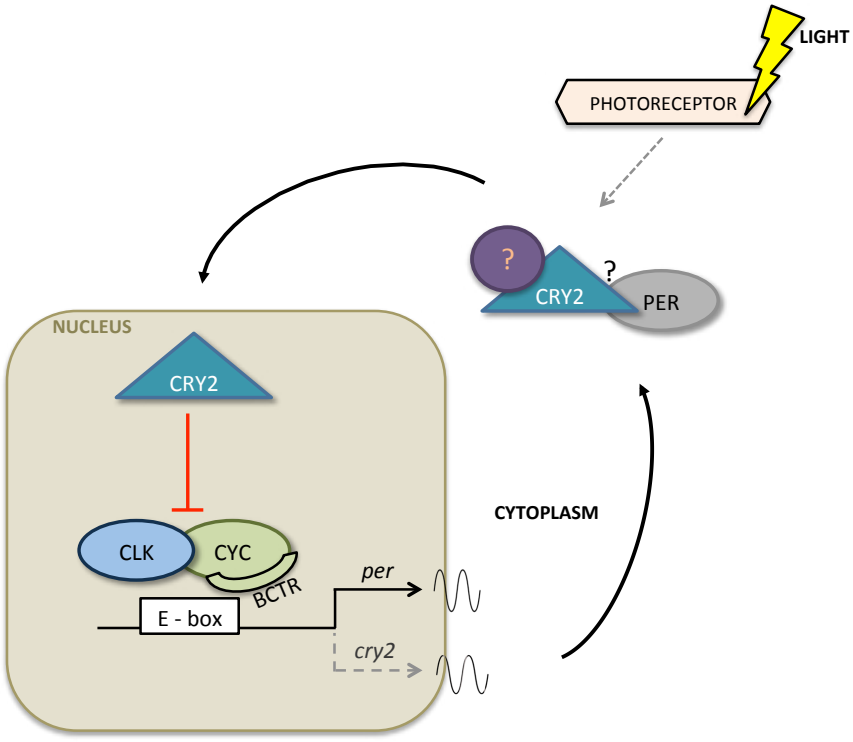


Figure 5.1. Model of the circadian clock in *Nasonia*. The main arm of the clock consists of a feedback loop where *Nv*CLK:CYC are the positive transcription factors driving the expression of *Nv*PER and potentially of *Nv*CRY2. *Nv*CRY2 is the main transcriptional inhibitor, repressing *Nv*CLK:CYC mediated transcription. *Nv*PER might bind to *Nv*CRY2 to regulate its function perhaps regulating nuclear entry as in mammals (Reppert and Weaver 2002) or stabilising CRY2 as in *Danaus* (Zhu et al. 2008). Light entrainment is not mediated by *Nv*CRY2, but through unknown photoreceptors.

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Summary

Adaptation to cyclical environmental conditions has led to the evolution of intricate time measuring mechanisms, known as biological clocks. Insects like other organisms, anticipate and respond to daily cycles via the circadian clock, and to seasonal changes by a photoperiodic mechanism. The precise details of these time mechanisms in various organisms are still subject to much research. Not much is known about the molecular basis of the circadian clock in insects other than *Drosophila melanogaster*. In this thesis, I investigate the clock of the parasitic wasp *Nasonia vitripennis* (Hymenoptera) that has strong light-driven behavioural rhythms and exhibits a seasonal response in the form of photoperiodic diapause.

The first step was to determine the characteristics of the *Nasonia* circadian clock and its synchronisation (entrainment) to light. *Nasonia* possesses orthologues of *Clock* (*Clk*), *cycle* (*cyc*), *cryptochrome2* (*cry2*), *period* (*per*) and *timeout* (*tim2*), but lacks *cryptochrome1* (*cry1*) and *timeless* (*tim1*) which are present in *Drosophila*. CRY1 is a blue-light sensitive photoreceptor that synchronises the clock with light stimuli from the environment in *Drosophila*, whereas CRY2 is a core clock protein (negative regulator) in the mammalian clock. I tested whether CRY2 may have a dual role as a negative regulator and photoreceptor in *N. vitripennis*, which would indicate a role in the circadian system.

Protein functional domains of *Nasonia* CRY2 (*Nv*CRY2) and other putative clock proteins CLK (*Nv*CLK), CYC (*Nv*CYC) and PER (*Nv*PER) were compared *in silico* between *Nasonia,* honeybee*, Drosophila* and the mouse. All the wasp proteins had higher sequence similarity to the honeybee and mouse than to *Drosophila* orthologues **(Chapter 2)**. This indicates that the transcriptional-translational feedback loop of the *Nasonia* clock is more similar to honeybee and mammals than to *Drosophila*, suggesting that CRY2 functions as a negative regulator in the circadian feedback loop of *Nasonia*, and not as a photoreceptor.

To study the clock mechanism of *Nasonia in vivo,* I examined the circadian oscillation in expression of putative clock genes over 24 hours, under both cycling and constant light conditions **(Chapter 2)**. I found that expression of *Nvcyc* mRNA oscillates over a 24 h time course in light-dark cycle, whereas that of *NvClk* does

not. Again, such an expression profile of *Clk* and *cyc* is more similar to honeybee and mammals rather than *Drosophila.*

I used the *Drosophila* Schneider (S2) cell system to study the negative feedback-loop of the *Nasonia* circadian clock, as there are no *Nasonia* cell lines available. A luciferase gene reporter assay was employed, allowing the transcriptional activity of putative *Nasonia* clock genes to be measured through the bioluminescent properties of luciferase. Presence of *Nv*CRY2 led to inhibition of transcriptional activity of *Nv*CLK:CYC heterodimer as monitored by diminished signalling of the reporter gene under PER promotor, confirming that *Nv*CRY2 is the main negative regulator of the clock feedback loop in *N. vitripennis.* It further revealed that *Nv*CRY2 acts as negative regulator independently of *Nv*PER*,* similar to the honeybee and mammalian circadian clock. *Nv*PER does not have the ability to inhibit transcriptional activity of *Nv*CLK:CYC alone, but whether *Nv*PER physically interacts with *Nv*CRY2 is yet to be determined. *Nv*PER could potentially regulate *Nv*CRY2 stability or nuclear localization.

To test whether *Nv*CRY2 also acts as a photoreceptor, I tested the light sensitivity of *Nv*CRY2 in an S2 cell luciferase assay **(Chapter 3)**. The *Nv*CRY2 negative regulator function was unaffected by exposure to light**,** indicating that *Nv*CRY2 is not degraded upon light exposure and thus not a light sensitive molecule. This excludes its role as a photoreceptor and implies that other light sensitive proteins are responsible for the entrainment of circadian rhythms in *Nasonia.*

The function of *Nv*CRY2 was also assessed *in vivo*. I used behavioural experiments based on locomotor activity, to investigate the role of *Nv*CRY2 in light-mediated entrainment of circadian rhythms **(Chapter 3).** As *Nv*CRY2 was found to be a transcriptional repressor of the circadian clock, I expected a profound change in locomotor activity in constant light conditions. However, neither the length of the free running period (rhythmic behaviour in absence of environmental stimuli such as light) nor the robustness of rhythmicity was affected upon *Nvcry2* RNAi

knockdown, suggesting that multiple, redundant regulatory mechanisms that maintain rhythmicity of locomotor may exist in *Nasonia.*

Additional experiments delivering light pulses and phase-shifted light dark cycles (jet lag) were employed to test the ability of *Nv*CRY2 to reset the clock **(Chapter 3)**. I found that *Nvcry2* knockdown in females did not affect the ability to respond to a 1 hour light pulse, but in males did attenuate the phase shift response. This suggests sex-specific differences in circadian clock regulation, light sensitivity, or the efficiency of RNAi knockdown. Intriguingly, *Nvcry2* knockdown did not affect the ability of males to re-entrain to a 6 hr advanced light-dark cycle. This re-entrainment is likely achieved via different photoreceptors and pathways, which are responsible for relaying light information to the clock. Based on these experiments, it can be concluded that *Nv*CRY2 does not play a key role in the light-entrainment of the circadian clock.

I next investigated which photoreceptor might be responsible for light entrainment of the circadian clock in *Nasonia*. Various light wavelengths were tested in 6 hr phase-advance or delay re-entrainment experiments to measure how quickly the wasps synchronise **(Chapter 3)**. In phase advance, both sexes entrained faster to longer wavelengths, especially ~ 515 nm. Conversely, the response to phase delay was more sensitive to shorter wavelengths (< 464 nm). This response might be regulated by UV-opsins or other photoreceptors, which have not yet been identified in *Nasonia*.

In **Chapter 4,** I analysed natural genetic variation to identify genes that are involved in regulation of circadian rhythms and seasonal response in *Nasonia*. I used the *Nasonia vitripennis* Genetic Reference Panel (NVGRP) that was derived from a genetically variable population. I found substantial variation between lines in diapause propensity (seasonal response) and free running activity period (circadian rhythm). A genome-wide association analysis (GWAS) identified several SNPs associated with diapause propensity in four genes on chromosome 4. The function and role of these four candidate genes in regulation of photoperiodic diapause is yet to be determined.

My study has led to a better understanding of the circadian clock and photoperiodic timing in insects. The autoregulatory feedback mechanism of *Nasonia* was found to be similar to honeybee*,* stressing the conserved role of clock genes in Hymenoptera and its similarity to the mammalian clock rather than *Drosophila.* The hypothesis that *Nv*CRY2 is a light-sensitive molecule that serves both as a photoreceptor and a negative regulator of the *Nasonia* circadian clock was not supported. Other, presumably visual cues and long wavelength photoreceptors seem to be responsible for light-mediated entrainment of the clock. Further research should aim to determine the identity and function of these visual photoreceptors in the circadian clock. Functional analysis of the SNPs and corresponding genes associated with diapause propensity, as revealed in my GWAS study, should help to uncover the genetic basis of seasonal timing in *Nasonia*.

Sumenvatting

Dutch translation by Anna Rensink

De adaptatie aan cyclische omgevingscondities heeft geleid tot de evolutie van complexe tijdsbewustzijnsmechanismen die ook wel bekend zijn als de biologische klok. Insecten kunnen, net als vele andere organismen, anticiperen en reageren op dagelijkse cycli en seizoensgebonden veranderingen door een fotoperiodiek mechanisme. De werking van dit mechanisme in verscheidene organismen is onderwerp van vele studies. De huidige kennis van de moleculaire basis van de circadiaanse klok in insecten is gebaseerd op *Drosophila melanogaster*. In dit proefschrift onderzoek ik de klok van de parasitaire wesp Nasonia vitripennis (Hymenoptera) welke zijn bioritme sterk aanpast aan het licht en een fotoperiodiek respons vertoont op seizoensveranderingen zoals het induceren van diapauze.

De eerste stap was het karakteriseren van de circadiaanse klok van *Nasonia* en zijn aanpassingsvermogen aan het licht. *Nasonia* heeft orthologen van *clock (clk), cycle (cyc), cryptochrome2 (cry2), period (per)* en *timeout (tim2),* maar in tegenstelling tot *Drosophila*, geen *cryptochrome1 (cry1)* en *timeless (tim1).* De fotoreceptor CRY1 in *Drosophila* is gevoelig voor blauw licht en synchroniseert de klok met lichtinvloeden uit de omgeving, terwijl bij zoogdieren CRY2 het belangrijkste klokeiwit is (als negatieve regulator). Ik heb getest of CRY2 in *Nasonia* mogelijk een duale rol heeft als zowel een negatieve regulator alsook een fotoreceptor*,* hetgeen een rol in het circadiaanse systeem zou suggereren.
De functionele domeinen van het klokeiwit CRY2 (*Nv*CRY2) en de andere potentiële klokeiwitten CLK (*Nv*CLK), CYC (*NvCYC)* en PER (*Nv*Per) zijn *in silico* vergeleken tussen *Nasonia,* de *honingbij (Apis), Drosophila* en de muis (*Mus musculus).* De eiwitten van *Nasonia* tonen meer overeenkomsten met de eiwitten van de honingbij en muizen dan met de orthologen van *Drosophila* **(Hoofdstuk 2)**. Aannemelijk is dat de transcriptie-translatie feedback loop van *Nasonia* meer overeenkomt met honingbijen en zoogdieren dan met *Drosophila* wat suggereert dat CRY2 functioneert als negatieve regulator in de klok van *Nasonia*, en niet als fotoreceptor.

Om het klokmechanisme van *Nasonia* in vivo te onderzoeken zijn de circadiaanse en oscillerende expressiepatronen van de potentiële klok genen bestudeerd gedurende 24 uur onder cyclische en constante lichtcondities **(Hoofdstuk 2)**. De expressie van *Nvcyc* mRNA oscilleert gedurende een 24-uurse licht-donker cyclus, terwijl *NvClk* niet oscilleert. Dit expressieprofiel van *Clk* en *cyc* komt meer overeen met honingbijen en zoogdieren dan met het expressieprofiel in *Drosophila.*

Om de negatieve feedback loop van de circadiaanse klok in *Nasonia* verder te bestuderen heb ik gebruik gemaakt van het *Drosophila* Schneider (S2) cel systeem omdat er geen *Nasonia* cellijnen beschikbaar zijn. De transcriptionele activiteiten van potentiële *Nasonia* klok genen zijn onderzocht met behulp van bioluminescentie in een luciferase reporter assay. De aanwezigheid van *Nv*CRY2 leidde tot een remming van de transcriptionele activiteit van het *Nv*CLK-CYC heterodimeer, gemeten als een verminderd signaal van het reportergen gedreven door een PER promotor. Dit bevestigt dat *Nv*CRY2 de belangrijkste negatieve regulator is in het klok mechanisme van *N. vitripennis.* Daarnaast laat het ook zien dat *Nv*CRY2 werkt als negatieve regulator, onafhankelijk van *Nv*Per, zoals dat ook is aangetoond in de circadiaanse klok van de honingbij en zoogdieren. *Nv*PER heeft niet het vermogen om de transcriptie activiteiten van *Nv*CLK:CYC te remmen, maar of *Nv*PER fysieke interactie aangaat met CYC moet nog worden bepaald. *Nv*PER zou mogelijk de stabiliteit of de nucleaire lokalisatie van *Nv*CLK:CYC kunnen reguleren.

Om te onderzoeken of *Nv*CRY2 ook als een fotoreceptor fungeert, heb ik de gevoeligheid voor licht van *Nv*CRY2 gemeten met behulp van een S2 cel luciferase assay **(Hoofdstuk 3)**. Blootstelling aan licht heeft de functionaliteit van *Nv*CRY2 als negatieve regulator niet beïnvloed, wat suggereert dat *Nv*CRY2 niet is afgebroken onder invloed van licht en dus geen lichtgevoelig molecuul is.

De functie van *Nv*CRY2 is ook in vivo onderzocht. De locomotor activiteit is gemeten tijdens circadiaanse ritmes op basis van lichtcycli, om meer inzicht te krijgen in de rol van *Nv*CRY2 op het gedrag **(Hoofdstuk 3)**. Omdat *Nv*CRY2 verondersteld wordt een transcriptionele repressor van de circadiaanse klok te zijn,

verwachtte ik een ingrijpende verandering in de locomotorische activiteit bij constant licht. Noch de lengte van de vrijlopende activiteit (ritmisch gedrag in afwezigheid van omgevingsstimuli zoals licht) noch de robuustheid van de ritmiciteit werd beïnvloed wanneer *Nv*CRY2 was uitgeschakeld (middels RNAi), wat suggereert dat er meerdere regulatiemechanismen bestaan in *Nasonia* die de ritmiciteit in locomotoractiviteit in stand houdt.

Aanvullend is onderzocht of het blootstellen aan lichtpulsen en faseverschuivingen van licht-donker cycli (jet lag) van invloed is op het vermogen van *Nv*CRY2 om de klok te resetten **(Hoofdstuk 3)**. Ik ontdekte dat, wanneer *Nv*CRY2 is uitgeschakeld, dit bij vrouwtjes geen verandering geeft in hun respons op een 1-uur durende lichtpuls, maar bij mannetjes toonde een faseverschuiving een verzwakt respons aan. Dit suggereert sekse-specifieke verschillen in de regulering van de circadiaanse klok, de lichtgevoeligheid of de efficiëntie van RNAi-knockdown. Intrigerend genoeg had de knock-out van *Nv*CRY2 geen invloed op het vermogen van mannetjes om te herstellen na een 6 uur durende licht-donkercyclus (periodiek). Mogelijk zijn er andere fotoreceptoren en routes verantwoordelijk voor het doorgeven van lichtinformatie binnen het klokmechanisme voor een dergelijk herstel. Op basis van deze experimenten kan worden geconcludeerd dat *Nv*CRY2 geen sleutelrol speelt in de op licht gebaseerde periodiek van de circadiaanse klok.

Vervolgens onderzocht ik welke fotoreceptoren mogelijk verantwoordelijk zijn voor de periodiciteit van de circadiaanse klok in *Nasonia*. Verschillende lichtgolflengten zijn getest gedurende 6 uur met versnelde- en vertraagde lichtfasen om te meten hoe snel de wespen synchroniseren **(Hoofdstuk 3)**. Bij een versnelde fase passen beide seksen zich sneller aan wanneer ze zijn blootgesteld aan een langere golflengte, voornamelijk bij ~515nm. Omgekeerd is de respons op een fasevertraging gevoeliger bij kortere golflengten (<464 nm). Deze respons kan worden gereguleerd door UV-opsinen of andere fotoreceptoren, die nog niet zijn geïdentificeerd in *Nasonia*.

In **Hoofdstuk 4** heb ik de natuurlijke genetische variatie geanalyseerd om genen te identificeren die betrokken zijn bij de regulatie van de circadiaanse ritmen en de seizoensgebonden responsen in *Nasonia*. Ik heb gebruik gemaakt van het '*Nasonia vitripennis* Genetische Referentiepaneel' (NVGRP) dat is gebaseerd op een genetisch variabele populatie van één geografische positie. Ik heb een aanzienlijke variatie gevonden binnen de diapauze respons (seizoensgebonden respons) en de vrijlopende activiteit (circadiaans ritme) tussen de lijnen. Een genoom-brede associatie analyse (GWAS) identificeerde verschillende SNP's die geassocieerd worden met diapauze binnen vier genen op chromosoom 4. De functie en rol van deze vier genen in de regulatie van fotoperiodieke diapauze moet nog worden bepaald.

Mijn studie heeft meer inzicht gegeven in de circadiaanse klok en de fotoperiodieke timing in insecten. Het zelfregulerende feedback mechanisme van *Nasonia* toont overeenkomsten met de honingbij en benadrukt de geconserveerde rol van klokgenen in Hymenoptera, en toont opmerkelijk meer gelijkenis met zoogdieren dan met *Drosophila*. De hypothese dat *Nv*CRY2 een lichtgevoelig molecuul is dat zowel fungeert als fotoreceptor en als een negatieve regulator van de circadiaanse klok is niet ondersteund. Vermoedelijk zijn andere visuele signalen en fotoreceptoren met gevoeligheid voor lange golflengten verantwoordelijk voor de lichtgevoelige periodiciteit van de klok. Verder onderzoek zou gericht moeten zijn op het karakteriseren en functioneren van deze visuele fotoreceptoren in de circadiaanse klok. Een functionele analyse op de SNP's en de aan diapauze geassocieerde kandidaatgenen uit mijn GWAS onderzoek zal een bijdrage kunnen leveren aan het ontrafelen van de genetische basis van de seizoensgebonden timing in *Nasonia*.

Acknowledgements

This PhD was a long journey, especially the writing up phase, which I was hoping to finish much earlier. Nevertheless, I am now finally writing this last part of the thesis. During my PhD, I have met many people whom I should thank and without whom I would not be able to accomplish this journey.

Firstly, I would like to thank my supervisors **Eran Tauber**, **Leo Beukeboom** and **Louis van de Zande** to give me this opportunity. Eran, thank you for your support through my PhD, many advises you gave me about the research and life lessons I will doubtlessly remember through my life. Hiking in Eingedi is definitely on the top of my life experiences. Louis, I will always be thankful for your friendly support and discussions on my results and believing in myself. I will never forget the moment, when I almost break door of your office when I discovered my first mutants. I also thank you for giving me exceptionally helpful comments on the manuscript. Leo, thank you for supporting me through the PhD and especially through the writing up phase. I really appreciate the patience you had while correcting the manuscript (I hope I will now remember the difference between Hymenoptera and hymenopteran).

I would like to thank to the members of the assessment committee **Ralf Stanewsky**, **Charlotte Helfrich-Förster** and **Bregje Wertheim** for their time to read and evaluate this manuscript. Thanks belong also to the defence committee members for accepting to take part in the PhD graduation ceremony.

Thank you my paranymphs, **Pina Brinker** and **Sylvia Gerritsma**, for helping me to finish my PhD journey. A big thank belongs to **Anna Rensink** for the translation of the Dutch summary and Publicity abstract in such a hurry.

Thanks to all members of the Marie Curie European Initial Training Networking group INsecTIME, which my PhD was part of. I have greatly enjoyed all the meetings, inspirational talks and discussions about chronobiology, science and life

in science. **Valeria Zonato**, you have special acknowledgement not only for your exceptional role as a manager, organising all the meetings to smallest details, but also for becoming very good friend with big soul and ears to listen. I cannot thank you enough. You become my big inspiration through my PhD for your adventurous nature, bravery and kindness. Big thanks to **Elena**, **Theresa** and **Jelena**, my colleagues in crime not only as a part of INsecTIME, but also our little *Nasonia* community. Elena thank you for answering my million questions about thesis and everything, your support was very important to me and I truly enjoyed discussing science with you. Good luck to you through your science journey. **Ane**! You deserve a special chapter for yourself. I cannot summarise all you mean to me with words. Thank you for being such an optimistic and supportive friend. The time in Leicester was great with you! I am giving you a big hug, stays printed indelible through the time. You worked so hard through the whole PhD and I wish you a lot of luck in your future career. Thank you **Lenka**, **Rossana**, **Faredin** and **Dora** for so much laughing we had together through all the meetings. **Enrico**, **Sanne**, **Emma** it was great to have you as a colleagues and friends.

I would like to thank everyone in Leicester who becomes part of my life there. Thank you, **Ezio Rosato,** for all your support and your enthusiasm about science. Thank you **Bambos Kyriacou** for your kindness and letting me to write up at university. **Helen Roe** without you nothing would be possible. Thank you **Laura Flavell** for becoming my friend and senior PhD to whom I could come to ask and discuss. **Nathaniel**, **Kam** and **Ben**, our small lunch group, thank you all for inside on British culture, humour and politics. **Mirko Pegoraro**, thank you for your mind of philosopher, you could always open my eyes to see the world from different angle. Thank you for showing me a lot of analysis and molecular techniques. I wish you the best of luck on your journey. **Giorgio Fedele,** you cannot possibly ever drink all the beer you were promised as an exchange for scientific support, troubleshooting, chemicals, endless lab experience and great knowledge of science, especially about *cryptochrome*. Thank you Giorgio, you definitely become my lab guru. **Lin**

Zhang, thank you for being such a supportive colleague. Thank you **Usha Aryal**, **Marta Scalzotto**, **Joana Branco Santos** and **Alex Hinks** for becoming such a good friends of mine.

Thank you everyone in Groningen who become a part my part of life through the time I spend there. Thank you **Anna Rensink** for everything, you were always there to help no matter whether it was molecular lab, *Nasonia* life cycle or my personal life trouble. Thank you **Rogier Houwerzijl** for all incubator arrangements. Thank you **Elzemiek** for all the advice and the roof above my head for several occasions, it was great to have you as a colleague and to share office with you. Thank you **WenJuan** and **Jessy** for all the little talks. **Yanli**, you are such a great person, I had a lot of fun with you doing all the sport activities, games and dinners. I hope you get a nice job soon and to see you more often in Amsterdam or anywhere else.

Ráda bych poděkovala své rodině, že mi byli oporou po dobu mého doktorského studia. Nebylo to vždycky snadné, ale vždy jste mě vyslechli a podpořili mě. Tati a mami, děkuju za všechno po domácku sušené ovoce, houby a med, bez kterého bych určitě nepřežila svůj pobyt v zahraničí. Děkuju Ivetko, za všechnu pomoc se stěhováním z místa na místo, hlavně do Holandska, kde jsi mi pomohla překonat můj první týden. Jaroušku a Amálko, že jste mi tak skvělými synovcem a neteří.
Děkuju I tobě Alenko Loužilová (Burianová), že jsi mi stála po boku celého mého universitiního života a zůstala mi skálopevnou přítelkyní i poté, co jsem odjela do zahraničí. Děkuji Zlatce Šustrové, za všechny společné výlety, které mi okořenily dobu doktorského studia a cestovní plány, které se snad ještě někdy uskuteční. Ivu Fornbaumová, děkuji, že jsi se mi stala blízkou kamarádkou a za všechna naše prožitá dobrodružství a cesty.

Lastly and most importantly, thank you **Daniel Maddison** not only for proof reading my thesis so many times, but mainly for becoming an environmental cue I can

entrain with. Without you, I would never approach this point of my PhD. Thank you for believing in me.